SOUVENIR

XIV Annual Convention of Indian Society For Veterinary Immunology & Biotechnology (ISVIB) and National Symposium on

Recent Trends In Immuno-Biotechnology Based Biologicals & Their Commercialization

{February 27-29, 2008}

Department of Veterinary Biochemistry &
Department of Veterinary Microbiology
College of Veterinary & Animal Sciences
G.B. Pant University of Agriculture & Technology
Pantnagar-263 145, U.S. Nagar, UTTARAKHAND

We gratefully acknowledge the generous contribution received from the following organizations towards sponsoring various events XIV Annual Convention of ISVIB and National Symposium held at Pantnagar from February 27 to 29, 2008.

- Indian Council of Agricultural Research , New Delhi
- Department of Biotechnology, New Delhi
- Department of Science & Technology, New Delhi
- Uttarakhand Council for Science & Technology, Dehradun
- State Biotechnology Programme, Haldi (Uttarakhand)
- Zydus AHL, Ahmadabad
- Nidhi Traders, Rudrapur
- Twomen's Business Organization, Haldwani
- Ventri Biologicals Vaccine Division, Pune
- Shipra Scientific Traders, Rudrapur
- Genetix Biotech Asia Pvt. Ltd., New Delhi

XIV Annual Convention
of

Indian Society For Veterinary Immunology & Biotechnology (ISVIB)

and
National Symposium
on

Recent Trends In Immuno-Biotechnology Based Biologicals And Their Commercialization

February 27-29, 2008

Souvenir

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&
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Pantnagar-263 145, U.S. Nagar, UTTARAKHAND

The souvenir is published on the occasion of XIV Annual Convention of Indian Society For Veterinary Immunology & Biotechnology (ISVIB) and National Symposium on "Recent Trends In Immuno-Biotechnology Based Biologicals And Their Commercialization" from February 27-29, 2008, organized by Departments of Veterinary Biochemistry and Microbiology, College of Veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar -263 145, Uttarakhand, India.

[The remarks and opinion expressed in the souvenir are entirely of the respective authors and does not in any case reflect the views of the organizing committee.]

Published by the Organizing Committee of the National Symposium on "Recent Trends In Immuno-Biotechnology Based Biologicals And Their Commercialization" XIV Annual Convention of Indian Society For Veterinary Immunology & Biotechnology (ISVIB) and National Symposium and type set by Mr. Akshay Swaroop Gupta, M/s Ocean Publication, Rampur and assisted by Mr. Mahesh Prasad, Junior Assistant and Mr. A.B. Sati, Lab Technician, Department of Veterinary Biochemistry, College of Veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar -263145, Uttarakhand,

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Archana Datta (Mukhopadhyay)
Officer on Special Duty (Public Relation)

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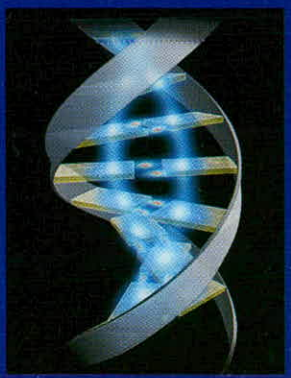
राष्ट्रपति भवन,
नई दिल्ली-110004
President Secretariat,
Rashtrapati Bhavan,
New Delhi-110004

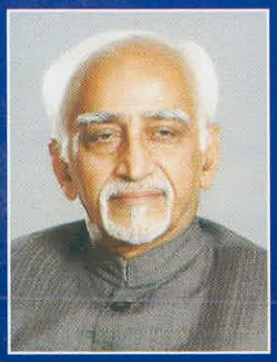
MESSAGE

The President of India, Smt Pratibha Devisingh Patil is happy to know that the G.B. Pant University of Agriculture & Technology is organizing the XIV Annual Convention of the Indian Society for Veterinary Immunology and Biotechnology and the National Symposium on "Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization" from 27th to 29th February, 2008 at Pantnagar.

The President extends her warm greetings and felicitations to the organizers and the participants and wishes the events all success.

Officer on Special Duty (PR)





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OFFICER ON SPECIAL DUTY
TO THE VICE PRESIDENT OF INDIA
नई दिल्ली/NEW DELH-110011
TEL ; 23016422/23016344 FAX : 23012645

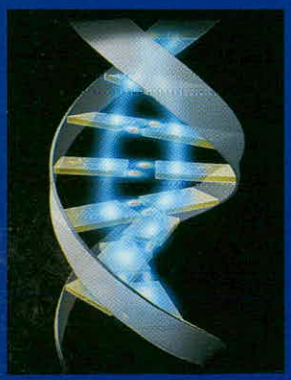
MESSAGE

Hon'ble Vice-President of India is happy to know that the G.B. Pant University of Agriculture & Technology is organizing the XIV Annual Convention of Indian Society of Veterinary Immunology & Biotechnology and National Symposium on "Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization" during February 27 - 29, 2008.

The Vice President extends his good wishes to the organisers and the participants for the success of the Annual Convention and the symposium.

(P. HARISH)

New Delhi
4th February, 2008



B.L. Joshi
Governor, Uttarakhand

सत्यमेव जयते

RAJ BHAWAN
Dehradun-248 003

February 11, 2008

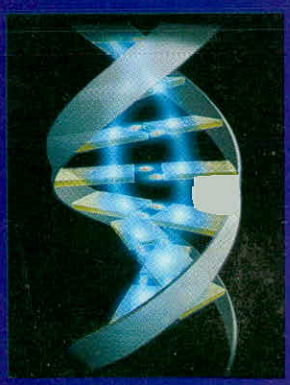
MESSAGE

I am glad to learn that the Indian Society for Veterinary Immunology and Biotechnology (ISVIB) and the College of Veterinary and Animal Sciences (G.B. Pant University of Agriculture and Technology, Pantnagar) are jointly organizing the Fourteenth Annual Convention of ISVIB and National Symposium on *"Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization"*, from 27th to 29th February, 2008.

Development of diagnostic, prophylactic and therapeutic products constitutes akey area of research in the sphere of veterinary biotechnology today. The immediate challenge before veterinary scientists in India is to research and develop processes for manufacturing these products indigenously, in a cost effective manner.

I am sure that this Symposium would provide an excellent opportunity to deliberate on crucial issues confronting researchers in the area of veterinary immunology and biotechnology. I convey my warm greetings to the organizers and participants and wish them every success in their endeavour.

(B.L. Joshi)



शरद पवार
SHARAD PAWAR

कृषि, उपभोक्ता मामले,
खाद्य और सार्वजनिक वितरण मंत्री
भारत सरकार
MINISTER OF AGRICULTURE
& CONSUMER AFFAIRS
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GOVERNMENT OF INDIA

11 February, 2008

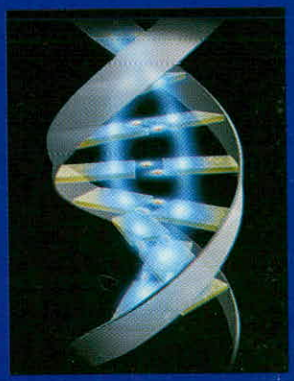
MESSAGE

I am delighted to learn that Indian Society for Veterinary Immunology & Biotechnology (ISVIB) and G.B. Pant University of Agriculture & Technology, Pantnagar are jointly organizing a National Symposium on "Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization" and the XIV annual convention of ISVIB at College of Veterinary & Animal Sciences, Pantnagar during 27 - 29 February, 2008.

The recent developments in Veterinary and Animal Sciences particularly Veterinary Immunology and Biotechnology have come up with great hope in terms of advancement of research and development and generation of wide range of biologicals. A substantial number of advanced technologies and biologicals have been developed in the frontier areas of Veterinary and Animals Sciences. In the era of global free trade, successful commercialization of the new technologies and biologicals is prime need of the hour. I hope that the deliberations of the symposium will help the scientists and planners to formulate better strategies for the future.

I wish the Annual Convention of ISVIB and the Symposium being held at Pantnagar, all success.

(SHARAD PAWAR)



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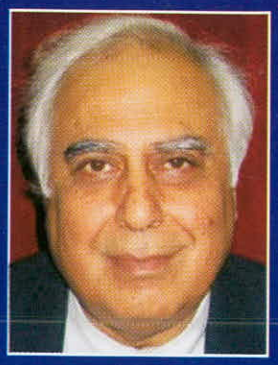
E-mail : sharadpawar.sp@gmail.com



कपिल सिब्बल
KAPIL SIBAL

विज्ञान एवं प्रौद्योगिकी तथा पृथ्वी विज्ञान मंत्री
भारत सरकार, नई दिल्ली
MINISTER FOR SCIENCE
& TECHNOLOGY
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GOVERNMENT OF INDIA

MESSAGE

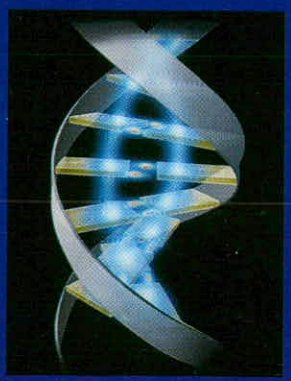
I am delighted to learn that Indian Society for Veterinary Immunology & Biotechnology (ISVIB) is organizing its XIV Annual Convention and a National Symposium on "Recent Trends in Immuno Biotechnology based Biologicals and their Commercialization" at College of Veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar, during 27-29 February, 2008.

The recent developments in the fields of Veterinary Immunology and Biotechnology offer a great hope in terms of advancement of research, development and production of wide range of biologicals. Various Modern technologies and biologicals have since been developed in the niche areas of Veterinary Immunology & Biotechnology. In the era of free trade, successful commercialization of the new technologies and biologicals is extremely important. I am sure that the discussions and resolutions of the symposium will pave the way for scientists and planners to formulate better strategies for the future.

I wish the convention and symposium a grand success.

Sibal

(KAPIL SIBAL)



डा. मंगला राय
सचिव एवं महानिदेशक
MANGALA RAI
SECRETARY & DIRECTOR GENERAL

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MINISTRY OF AGRICULTURE, KRISHI BHAVAN, NEW DELHI 110 001
TEL : 23382629; FAX : 91-11-23387293; E-MAIL : mrai.icar@nic.in

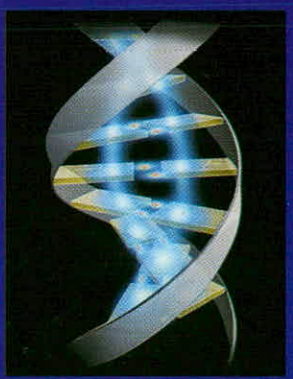
MESSAGE

It gives me great pleasure to know that the Indian Society for Veterinary Immunology & Biotechnology (ISVIB) is organizing its XIV Annual Convention and a National Symposium on "Recent Trends in Immuno Biotechnology based Biologicals and their Commercialization" at College of Veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar, during 27-29 February, 2008.

India has immense animal wealth in terms of population and germ plasm, but the potential of animal industry still remains untapped. Sustainable growth and development in livestock sector is the only solution to the problems of over stressed agriculture, unemployment and poverty. To overcome these problems the focus of research should be towards development of commercially viable diagnostics, prophylactics and therapeutics to maximize the animal production through improved health. The theme of the symposium, therefore, is timely in the era of globalization of economy. I hope that the deliberations would be helpful in formulation animal health strategies for the control of Livestock and poultry diseases.

I wish the convention and symposium a grand success.

(MANGALA RAI)



Dr. S.K. Bandyopadhyay
Commissioner, Animal Husbandry

भारत सरकार
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पशु पालन, डेयरी और मत्स्यपालन विभाग
कृषि भवन,, नई दिल्ली-110 001

GOVERNMENT OF INDIA
MINISTRY OF AGRICULTURE
DEPARTMENT OF ANIMAL HUSBANDRY, DAIRYING & FISHERIES
KRISHI BHAVAN, NEW DELHI 110 001

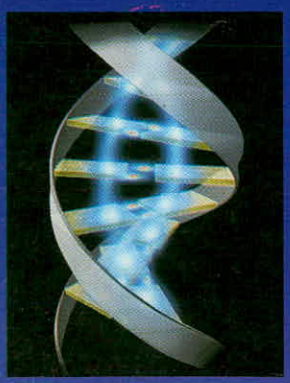
MESSAGE

I am happy to learn that Indian Society for Veterinary Immunology & Biotechnology (ISVIB) is organizing its XIV Annual Convention and a National Symposium on "Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization" at College of Veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar from 27th to 29th February, 2008.

In spite of having large livestock population in the country and productivity in terms of milk or meat is poor. One of the reasons for below par production is the presence of a number of livestock diseases in the country. Some of the diseases of livestock are also a potential threat to human life. Timely diagnosis of these livestock diseases and availability of appropriate prophylactics are two vital necessities for control and containment of livestock diseases. Therefore research endeavor on livestock health should focus primarily on development of cost effective and easy to apply veterinary biologicals. I am confident that presentations and deliberations of the symposia will be able to focus on these two vital components of livestock health management.

I extend my best wishes to the participants and organizers and hope that the convention and the symposium are a grand success.

(Dr. S.K. Bandyopadhyay)



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सं० 8208/VIP/08
दिनांक 08/02/2008

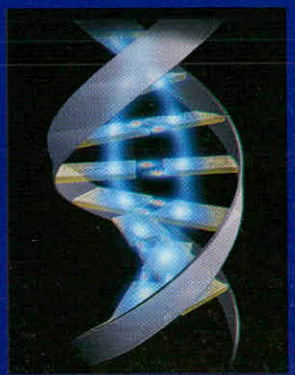
MESSAGE

I am delighted to learn that Indian Society for Veterinary Immunology & Biotechnology (ISVIB) is organizing its XIV Annual Convention and a National Symposium on "Recent Trends in Immuno Biotechnology based Biologicals and their Commercialization" at College of Veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar, during 27-29 February, 2008.

The recent developments in the fields of Veterinary Immunology and Biotechnology offer a great hope in terms of advancement of research, development and production of wide range of biologicals. Various Modern technologies and biologicals have since been developed in the niche areas of Veterinary Immunology & Biotechnology. In the era of free trade, successful commercialization of the new technologies and biologicals is extremely important. I am sure that the discussions and resolutions of the symposium will pave the way for scientists and planners to formulate better strategies for the future.

I wish the convention and symposium a grand success.

(Trivendra Singh Rawat)



Dr. A.P. Sharma
Vice-Chancellor

G.B. Pant University
of Agriculture & Technology
PANTNAGAR-263145
Distt. Udham Singh Nagar (Uttarakhand) INDIA

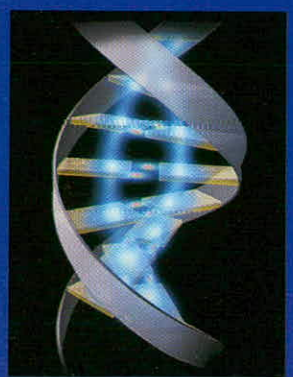
MESSAGE

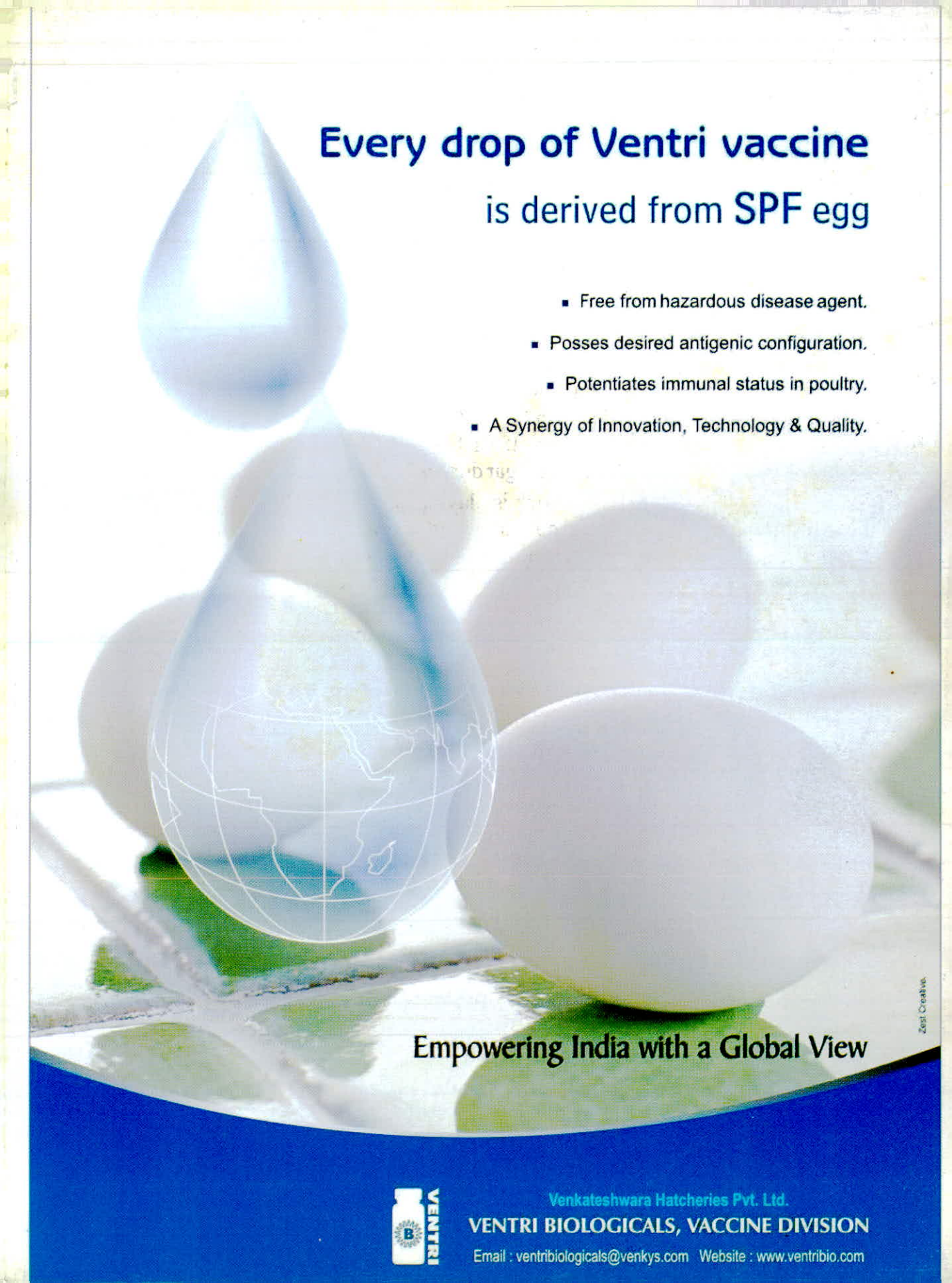
I am delighted to know that Indian Society for Veterinary Immunology & Biotechnology (ISVIB) is organizing a National Symposium on "Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization" and the XIV annual convention of ISVIB at College of Veterinary & Animal Sciences, Pantnagar during 27 - 29 February, 2008.

Livestock is playing an important to economic growth in developing countries and the application of biotechnology is largely dictated by commercial considerations and socio-economic goals. Using technology to support livestock production is an integral part of viable agriculture in multi-enterprise systems. Molecular markers are increasingly being used to identify and select particular genes that lead to the desirable traits and it is possible to select superior germplasm and disseminate it using artificial insemination, embryo transfer and other assisted reproductive technologies. These technologies have been used in genetic improvement of livestock and accordingly the economic returns have been significant. However, these animals are prone to many diseases particularly infectious ones and lead to high rate of economic losses. Therefore, the major thrust of animal biotechnology at present is the production of cheap and cost-effective diagnostic kits and vaccines. The organizers have taken the theme timely and I hope that the deliberations of the symposium shall be beneficial to the scientists, students, potential entrepreneurs and the livestock farmers.

I extend warm greetings and felicitations to the organizers and participants and wish the Symposium a grand success.

(A.P. Sharma)
Vice-Chancellor

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XIV Annual convention of Indian Society for Veterinary Immunology & Biotechnology. (ISVIB) and National Symposium on Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization

February 27-29 2008

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Dr. A.K. Ghosh , Assoc. Prof., GAB	Member
Dr. Shingini Sharma, Asstt. Prof., VBC	Member
Dr. Anita, Asstt. Prof., LPT	Member
Dr. Sarita Jena, P.G. Student, VBC	Member
Dr. Devki Pilkhwal , P.G. Student, ABT	Member

Poster Session Committee

Dr. V.S. Rajora, Prof., VCM	Chairman
Dr. Anil Kumar, Prof., VNN	Member
Dr. Ishwar Singh, Prof, VAN	Member
Dr. P. Prabhakaran, Assoc. Prof., LPT	Member
Dr. Anu Rahal, Asstt. Prof., VPT	Member
Dr. Rajeev Ranjan, Asstt. Prof., VPA	Member
Dr. Rahul Sati, P.G. Student, ABT	Member

GOBIND BALLABH PANT UNIVERSITY OF AGRICULTURE AND TECHNOLOGY: AN INTRODUCTION

University Education Commission (1949) headed by Dr. S. Radhakrishnan was constituted by Government of India for development and upliftment of education. The recommendations of this commission resulted in the establishment of rural universities to impart agricultural education among rural people. The first Joint Indo-American team constituted by Government of India (1955) studied the future needs of agricultural education and opted for land grant system for opening Agricultural Universities. Government of Uttar Pradesh and Indian Council of Agricultural Research initiated establishment of University at Tarai State Farm, Haldi in collaboration with the University of Illinois, USA. Pt. Govind Ballabh Pant, the then Home Minister, Government of India and Major H. S. Sandhu, the then General Manager of the erstwhile Tarai State Farm, Haldi played an instrumental role in establishment of agricultural university. As a result of their efforts, the first and foremost agricultural university of India, 'UP Agricultural University' came into existence by an act of Legislation, UP Act XLV of 1958 on Tarai State Farm in foothills of Himalayas with an area of 16,000 acre and a perimeter of 28.52 km. The University was dedicated to the Nation on November 17, 1960 by the first Prime Minister of India Late Pt. Jawaharlal Nehru. After amendment of Uttar Pradesh Universities Re-enactment and Amendment Act (1974), 'UP Agricultural University' was renamed as Govind Ballabh Pant Krishi Evam Prodyogik Vishwa Vidyalaya, Pantnagar.

During last 47 years of its legendary journey, the University has grown up as premier institute of higher education in Agriculture, Technology and allied streams leading to internationally renowned model for research and extension in frontline areas. The University is regarded as harbinger of Green Revolution. This is the only Agriculture University in the country which has been awarded with Sardar Patel Outstanding ICAR Institution Award twice by Indian Council of Agricultural Research in the year 1997 and 2005.

The mandate of the University is to make provision for the education of the rural people of Uttarakhand in different branches of study, particularly agriculture, rural industry and business, and other allied subjects and undertaking field and extension programme. The University has committed itself to all round agricultural development in the country through its concerted efforts and innovative education, research and extension programs.

The university was developed with a common campus for all the constituent colleges and a residential built-up for university employees. The University offers 16 undergraduate, 73 masters and 55 Ph.D. programmes managed by 76 departments. This is the only agricultural University having a separate faculty for engineering with 8 branches.

Organizational Set-up

The University is headed by the Board of Management with Vice-Chancellor as its ex-officio chairman and Secretaries of Govt. of Uttarakhand pertaining to Finance, Agriculture and Education as its ex-Officio members. ICAR, Legislative Assembly of Uttarakhand and Government of Uttarakhand also have their nominees in this body. The Vice-Chancellor is vested with the executive authority for all the matters to be dealt with on day to day basis. Each college is headed by a senior faculty member as Dean of the college who report to the Vice-Chancellor related to all the matters pertaining to the college. Each college has variable number of departments which are headed by Head of the Department and are answerable to the Dean of the College. It has been a

constant endeavor to upgrade the entire UG and PG syllabus to impart latest information in different subjects. This is being taken care off by the faculty and monitored by the system of feed back mechanism at central level by the registrar.

Academic Profile

The University started its first academic session on July 9, 1960 with 245 students. Initially the university had College of Agriculture, College of Veterinary Medicine and School of Basic Sciences and Humanities. To keep pace with the growth and development of the society and also considering the needs and priorities for the upliftment of agrarian population, the university has established following 10 colleges till date:

College	Department	U.G.	Masters*	Ph.D.*
Agriculture	11	02	13	13
Home Science	05	01	04	03
Veterinary & Animal Sciences	17	01	24	20
Basic Sciences & Humanities	10	-	11	08
Technology	10	08	13	09
Agribusiness Management	05	-	03	01
Fishery Sciences	05	01	02	01
Forestry & Hill Agriculture	07	01	03***	-
Horticulture**	06	02	-	-
Post-Graduate Studies*				
Total	**76**	**16**	**73**	**55**

* Post-Graduate programmes of all the colleges are coordinated and monitored by College of Post-graduate Studies.

** Also offers Certificate Course in Horticulture Management Diploma in Commercial Horticulture.

***M.Sc. Ag. (Horticulture) and M.Sc. Ag. (Vegetable Sc.) programmes are being run both at College of Agriculture, Pantnagar and College of Forestry & Hill Agriculture, Ranichauri.

Initiation Year of Different Programmes

Programme		Year
Undergraduate		
1.	B.Sc. Ag. & A.H. / B.V.Sc. & A.H.	1960
2.	B. Tech. Agril. Engg.	1962
3.	B. Tech. Civil/Electrical/Mechanical Engg.	1966
4.	B.Sc. (Pure Science)*	1968
5.	B.Sc. Home Science	1971
6.	Diploma in Home Science*	1971
7.	B. Tech. Computer/Electronics/Communication/Production Engg.	1983
8.	B. F. Sc	1985
9.	B.Sc. Forestry	1986
10.	B. Tech. Information Technology	2001
11.	Diploma in Commercial Horticulture	2004

12.	B.Sc. Horticulture	2005
13.	B.Sc. Food Technology	2006

Masters

1.	M.Sc. Ag. / M.Sc.	1963
2.	M.V.Sc.	1964
3.	M. Tech. Agril. Engg.	1968
4.	M. Tech. Others	1971
5.	M.Sc. Home Science	1978
6.	M.F.Sc	1996
7.	M.B.A. (Agribusiness)	1998
8.	M.B.A. (Technology)	2006
9.	M.C.A.	2006

Ph.D. 1966

Certificate Courses

1.	Horticulture	2004

* Now discontinued

To enrich students with diverse aptitude of science and arts, liberal education is also imparted making it an integral part of professional degree programmes. Visualizing the contribution of rural women population and to strengthen their role in various development programs, 20% seats are reserved for rural girls.

Extension education

The university has full-fledged Directorate of Extension Education to carry out various extension activities in the state of Uttarakhand. The activities of the Directorate are spearheaded by Extension Advisory Committee with Vice-Chancellor as a Chairman and Director Experiment Station and Dean of Colleges as its members and Director Extension Eductaion as its member-secretary. The main objective of this directorate is to disseminate the latest technologies emerging out of research laboratories in its area of responsibility. It also aims at identifying the problems of farmers, initiating need-based and problem-oriented research for enhancing production and improving economic condition of rural mass in the State. The directorate has 11 Krishi Vigyan Kendras. Besides these, the directorate is having two Krishi Gyan Kendras in the State. Directorate of Extension organizes various extension education programmes through farm advisory services like organization of Veterinary Extension Programmes, Home Science Extension Programmes, Farm Machinery Extension Programmes, Integrated-Village Linkage Programme (IVLP), All India Farmers' Fair and Agro Industrial Exhibition at Agricultural Technology Information Centre (ATIC) to provide information to the farmers of the state and solve their agricultural problems. The directorate through its various arms has been organizing trainings through out the year for officers representing government and public undertakings, farmers, farm women and unemployed youth. Technology dissemination is achieved through radio broadcast, TV programme, frontline demonstration, exhibitions and participation in national and international Agri-Expos. It also publishes and distributes literature by releasing two monthly magazines, viz., Indian Farmers' Digest in English and Kisan Bharti in Hindi as well as vocational books related to technologies developed. A telephonic helpline for farmers has been established where they can get in contact

with experts of relevant field for solution of their problems.

Research

The Directorate of Experiment Station coordinates the entire research in the University with the co-operation of the Dean of all Colleges. A research advisory committee with the Vice-Chancellor as its chairman, Directorate of Experiment Station as its Secretary and Dean of different Colleges as its members decide policies regarding the research work to be undertaken. All the research projects whether funded by outside agencies or university funded are being run through this directorate.

Research Breakthrough

The field of vegetables, horticulture, agroforestry and flowering plants has been richly contributed by the university with development of total 204 new varieties. Site Specific Nutrient Management (SSNM) for rice-wheat cropping system has been developed along with cultivation of rice crop through direct seeding. High Density planting in Mango has been successfully implemented. The millet based food products including baby food, production of Jaggery Chocolate and Jaggery Storage Bin has been developed in the field of food technology. Research breakthroughs like development of vaccine for the control of salmonellosis in chicks (Toxoid vaccine), kit for the detection of urea adulteration in milk, diagnostic kit for the detection of pregnancy in cattle and buffalo on 18th day of breeding, Embryo Transfer Technology (ETT) in animals, inclusion of herbs as feed additives in dairy animals, improvement of poor quality roughages by urea treatment, development of complete feed block, development of urea molasses mineral block, identification of brain centers responsible for rumeno-reticular movements, development of cost effective immunomodulators are some of the contributions of animal scientists. In the field of Agricultural Engineering, university has developed and to some extent commercialized 'Pant' Zero Till Ferti Seed Drill, improved Hill implements like Nasuda, Hill plough, Danala, Damala, paddy thresher, horticultural hand tools, animal yoke and 'Pant' Animal Drawn Zero Till Drill, 'Pant' Tarai Biogas Plant, 'Pant' Insulated Biogas Plant, 'Pant' hardened hoof shoe for draught animals. Technology for artificial aeration and showering system in breeding ponds for successful spawning of carps even without rains and monsoon has been developed for fish keepers. Improved method of vermi-composting has been developed.

University Library

Established in 1960, the University Library has a magnificent multistorey structure situated opposite the Administrative Building with nearly 3.6 lakh volumes of book, 421 journals, 120 online journals and seating capacity for 500 readers at a time. The library is computerized and can access to various books and journals through internet. Bibliographic information of all the documents of the library is digitized. Presently library is equipped with 25 PCs, database server, web server, digital library server, CDH-server, printers, etc. which is operated under Linux based local area network. The library subscribes various bibliographic databases (i.e. CAB-CD, AGRIS, FSTA, BIOSIS, AGRICOLA, COMPENDEX) and reference sources in CD-ROM form. The library web OPAC (on-line public access catalogue), online journals and other online information sources are searchable through library web site at http://202.141.116.194/. It has 256 kbps internet connectivity exclusively for library. Local area network of the library is connected with campus network through fiber optics line. Library operates a Rental Text Books scheme under which students can borrow up to 10 or more books in a semester at nominal rent.

Students Welfare

The Students Welfare is looked after an officer of the university Dean, Student Welfare who organizes programmes like orientation, advisement and counseling of students and warm reception of new students every year. The stay facility to house more than 3500 students is provided in 18 hostels which are having independent cafeterias that are run by the students themselves on no-profit-no-loss basis. The university looks after the infrastructure and financial management part of the cafeterias only. For providing medical and health facilities a full fledged 20 bedded hospital is functional within campus. To enrich the students with interpersonal skills and recreational activities 11 professional societies and 17 games and sports clubs are actively engaging the students in various activities of their interest. For meeting the demands of needy student's, financial assistance of over Rs. 19.0 lakhs is being provided to the students as scholarship every year. DSW is responsible for monitoring the students discipline both faculty wise as well as at central level.

Directorate of Placement & Counselling

It functions under a Director of Placement and Counselling and is responsible for arranging job opportunities for the graduates and post-graduates of the University by inviting employing agencies from public and private sectors to the campus. The Directorate has state-of-the-art facilities for selection procedure.

Communication Facilities

University has well-equipped Communication Centre for media production and services, media centre for publicity and maintaining liaison with print and electronic media, printing press, audio and Video film production facilities for broadcast and telecast and cable T.V. production and screening for campus residents.

University Offers

1. Consultancy to the industries and entrepreneurs in the areas of food technology, tissue culture, bio-pesticides production, mushroom production, civil and construction work, etc. through inter-sectoral collaboration and custom-built services.
2. Plant and animal disease diagnosis and treatment at Plant Clinic and Animal Clinic of the University.
3. Training to administrators, managers, trainers, teachers and professionals.
4. Training to extension officers of third-world countries.
5. Training for self-employment in fisheries, mushroom culture, bee-keeping, poultry farming, swine production, Angora rabbit farming, livestock product management, etc.
6. Service to the industries through analysis for pesticide, heavy metals, microbes, toxicants and other pollutants.
7. Production and distribution of seeds, plant saplings, spawns for mushroom production, fingerlings, bio-fertilizers, bio-pesticides, agriculture implements, etc. at reasonable cost.
8. Testing of soil and water samples.
9. Supply of heifers of cross-bred cows.

Other Facilities/functions of the University

1. State agricultural management and extension training Institute (SAMETI), Uttarakhand has been established to provide HRD support in innovative areas.
2. Agricultural Technology Management agency (ATMA) has been established in each district as an autonomous institution providing flexible working environment involving all the stakeholders of district development.
3. Joining hands with private organization like Jaikisan, MCX and Yes Bank for better technology transfer.
4. Knowledge partner for development of Uttarakhand Agri-Portal.
5. Working as consultant for tell agriculture with Himalayan Institute Hospital Trust in process of establishing Village Resource Centre at different out stations and KVKs.
6. Proposal of Kisan Sewa Kendra has been submitted to the state government so as to reach farm families, which are away from KVKs.
7. Telephone helpline is available to farmers on 05944–234810.

COLLEGE OF VETERINARY AND ANIMAL SCIENCES: PAST, PRESENT AND FUTURE

Establishment

The College of Veterinary Medicine came into existence in 1960 as one of the two constituent colleges of the first agricultural university of India "UP Agricultural University," which was later renamed as Govind Ballabh Pant Krishi evam Prodyogik Vishwa Vidyalaya in the year 1974.

The College started imparting veterinary education by enrolling first batch of 100 students on July 9, 1960 for B. V. Sc. & A.H. degree program. Department of Physiology and Pharmacology was one of the four departments that constituted the college. The other three were Department of Anatomy and Histology, Department of Pathology and Hygiene, and Department of Surgery and Medicine. The college of Veterinary Medicine was then renamed as College of Veterinary Sciences in the year 1974 and in 2003 it was renamed as College of Veterinary and Animal Sciences after inclusion of departments of Animal Sciences from College of Agriculture.

Mission and Goals

The college was established with the following mandates:

a) To impart undergraduate and postgraduate education for producing competent veterinarians.

b) To conduct basic and applied research on various aspects of livestock health, production and livestock products technology.

c) To carryout veterinary and animal husbandry extension activities through farm advisory services, health care services, organizing animal welfare camps, livestock shows etc.

d) To provide expert advice in veterinary and animal husbandry matters including diagnostic services and health coverage programs.

Current Mission

The current mission of the College of Veterinary and Animal Sciences is not different from the original set forth at the time of establishment except that the focus has now shifter to the new state of Uttarakhand, which came into existence in November 2000. Based on the current needs of the changing Veterinary and Animal Husbandry scenario at the national and international levels, and needs of user agencies and stakeholders, the education and research programs are being reoriented from time to time.

Goals

Keeping the mandate in view, the College of Veterinary and Animal Sciences has set itself the following goals:

1. To create opportunities for quality education in different disciplines of Veterinary and Animal Sciences.
2. To generate technologies to improve the productivity of livestock, provide better animal health coverage and assist rural youth, men and women to adopt improved technologies.

3. To provide the required assistance to the planning and development departments of the state government and other agencies to test these improved technologies, fine tune and disseminate them for the benefit of the farmers.
4. To strengthen and improve the infrastructure for providing quality education by constantly improving the syllabi of undergraduate (UG) and Post-graduate (PG) courses.

These goals are directed towards making the College of Veterinary and Animal Sciences as one of the best in the country for providing education and training to the students in the formal stream, generate appropriate technologies, evaluate them for adoption and make them accessible to the farmers.

Development of the College

College of Veterinary Medicine was reorganized in 1974 and re-designated as College of Veterinary Sciences to meet the demands of the society and to keep pace with the developments in various spheres of veterinary sciences. The department of physiology and Pharmacology was bifurcated into Department of Pharmacology and Toxicology and Department of Physiology. Further some new departments were also created and the total number of units went up nine. In the year 1992, the Department of Bacteriology and Public Health was bifurcated into Department of Microbiology and Department of Public Health. At this time the college had 10 departments and one independent unit i.e. Veterinary Teaching Hospital. The guidelines and recommendations of Veterinary Council of India (VCI) for Minimum Standard of Veterinary Education catalyzed reorganization and different animal husbandry and technology subjects were brought under the umbrella of veterinary sciences. So a total seventeen departments were created in the college in 1996 to incorporate new trends in veterinary sciences and boost research in the cutting edge technology to enhance livestock productivity. The college has now seventeen departments and three independent units:

1. Department of Veterinary Anatomy
2. Department of Animal Nutrition
3. Department of Animal Reproduction, Gynaecology and Obstetrics
4. Department of Clinical Veterinary Medicine, Ethics and Jurisprudence
5. Department of Epidemiology and Preventive Medicine
6. Department of Genetics and Animal Breeding
7. Department of Livestock Production and Management
8. Department of Livestock Products Technology
9. Department of Veterinary Microbiology
10. Department of Veterinary Parasitology
11. Department of Veterinary Pathology
12. Department of Veterinary Pharmacology and Toxicology
13. Department of Veterinary Physiology
14. Department of Veterinary Surgery and Radiology
15. Department of Veterinary and Animal Husbandry Extension
16. Department of Veterinary Biochemistry
17. Department of Veterinary Public Health

The three independent units are:

1. Veterinary Teaching Hospital.
2. Animal Biotechnology Centre
3. Animal Disease Diagnostic Centre.

Infrastructure

The physical facilities of the college comprise of classrooms, laboratories, committee rooms, conference hall and an auditorium. Each department in college has well equipped UG and PG labs and a seminar/committee room. The laboratories are equipped with modern equipments and instruments required for basic and applied research. In recent years, the college developed some specialized laboratories to undertake research projects. The existing laboratories and lecture halls have been renovated and refurbished as per the present requirements. The college has sophisticated instrumentation facilities such as Electron Microscope, Computerized Electroencephalograph, Atomic Absorption Spectrophotometer, UV spectrophotometer, HPLC, PCR, Multimedia Projection, Ultrasound scanner, Geldoc system, Anaerobic chamber, ELISA readers etc. The ARIS Cell with Internet facilities and Veterinary Information Center have been established. All the faculty members have computer with internet facility. Seven new departments viz., Department of Animal Nutrition, Department of Genetics & Animal Breeding, Department of Epidemiology and Preventive Medicine, Department of Livestock Production & Management, Department of Livestock Products Technology, Department of Veterinary & Animal Husbandry Extension and Department of Veterinary Biochemistry were added to the College in 1996, bringing the disciplines of Veterinary and Animal Husbandry under one umbrella for evolving an integrated approach to education, research and extension activities. The IIIrd phase of the Clinical Complex including a modern auditorium with a seating capacity of 299 was also completed in 1996. A pig-cum-duck-cum-fish farm was also added to promote integrated animal farming. The college existed in the original building for a long time but with the creation of new departments and units, the shortage of working space was acutely felt. To overcome this, a new wing for housing the department of Veterinary Public Health was also constructed. A new building to accommodate seven new departments and Instructional Animal Farm were added in 2002. Similarly an Animal Instructional Farm comprising of a new block of Department of Livestock Production and Management and separate housing facilities for keeping horse, sheep, goat, pig and rabbit have been constructed besides semen production center and a large amphitheatre. This new complex was inaugurated on 17th November 2002 by His Excellency Sh. Surjeet Singh Barnala, Governor of Uttaranchal. A library for use of Veterinary students and staff has been established at college level with latest textbooks, reference books and scientific and semi-technical magazines.

Teaching

In order to strengthen academic programs, the college switched over from trimester system to semester system of education from session 1985-86, subsequently the college was amongst the leaders in introducing VCI system of examination in the year 1994. To strengthen veterinary teaching, the Livestock Research Centre and Poultry Research Centre were shifted under the administrative control of the Veterinary College in 2002 and subsequently re-designated as Instructional Dairy and Poultry Farms for effective training of the students. During last 47 years the college has made tremendous progress in teaching, research and extension.

UG Teaching

For proper distribution of lecture and laboratory classes, a Master Timetable has been prepared. Teaching Coordination Committees which include all teachers associated with teaching of particular batch, two students and a senior professor as Chairman, one for each batch of B. V. Sc. & A.H. degree program, have been constituted for proper implementation and monitoring of academic policies and redressal of problems, if any, in consultation with the Dean. For more effective teaching liberal use of audio-visual aids is being done. For this all the lecture rooms have been provided with overhead projectors and two lecture rooms have been provided with LCD projector and a computer with peripherals. As per the VCI guidelines the students have to complete 83 courses of 194 credit hours with an average of 21.55 credit hours load per semester. The lecture to laboratory class ratio is 104:90 thus more emphasis is laid on practical exposure. The curriculum comprises 22.16% basic, 24.23% production, 18.56% preventive, 27.83% therapeutic, 4.12% public health and 3.1% technology subjects. With a view to infuse work culture and emphasize on the dignity of labour, Work Programme have been additionally included in the prescribed UG curriculum. To impart training and learning by doing, the 'Earn While You Learn Programs' like practical poultry production, practical goat keeping, practical pig production and practical pet keeping are also operating. This program helps in not only learning the practical experience and to start their own enterprise after graduation but also enable them to earn part of their living. These programs are over and above the minimum standards of Veterinary Education guidelines of VCI. The revised curriculum provides need-based thrust on production courses and judicious reorganization of clinical courses is helpful in honing the practical skills. To give the students the feeling of society welfare, college has embarked on the national policy of rural upliftment through animal welfare by launching National Social Service (NSS) since 1967. It was made an essential part of the curriculum of B. V. Sc. and A.H. program since 1969-70 as a two credits course. Further to strengthen the practical aspects of equine training, a unit of NCC (R&V) has been started in the college since 2003. Continuing education program by invited lectures on the topics of general and academic interests are frequently arranged so that the students as well as staff of the college is kept abreast of latest developments. For preventing cruelty against animals and alleviate their suffering and create awareness and love and affection for the animals, Blue Cross Society registered with Animal Welfare Board of India, is in operation. With the constant encouragement, guidance and hard work, the students of this college have been securing good number of Junior Research Fellowships at national level during last five years in the competition organized by ICAR.

Post-graduate Program

Though administratively the PG programs are run under Dean PGS, the college is the functional unit through which various PG programs are executed. Veterinary Physiology was the amongst first department to start Masters program way back in the year 1964 while it was the only department to offer Ph. D. program in the year 1967. The number of Masters Programs offered rose from 5 in 1964 to 24 in 2007. Similarly, the Ph. D. programs offered shot up from one in 1967 to 20 in 2007. Each department has excellent facilities for post-graduate teaching and research. Post-graduate students generally select the problem for their research work from the ongoing research priorities of the concerned department. Almost all the post-graduate students get financial support either through the research grants in the form of fellowship or research assistantship. Departmental, faculty and research seminars are a regular feature and it is imperative for all the students and staff to attend the same. College has so far produced **2443** B. V. Sc. & A.H. student, **443** M. V. Sc. and **129** Ph.D. students.

Research

The research activity of the College started way back in 1962. It received further momentum with the launch of post-graduate programs in some of the disciplines viz. Gynecology & Obstetrics, Microbiology, Pharmacology & Toxicology, Physiology and Surgery & Radiology during the year 1964. The establishment of air-conditioned Semen Biology Laboratory and Tissue Culture Lab during the year 1968 was another milestone in the field of research. A modern and well equipped necropsy building was added to the college in the year 1970. An Electron Microscopy lab as a central facility has been established in the year 2006 to carry out advanced research. With an aim to give new impetus to animal health and production, need-based and problem-oriented research thrust areas have been identified. Some of the identified areas of cutting edge technology in different disciplines are ethno-medicine, acupuncture, electrophysiology, vaccinology, immunodiagnostics, disease surveillance and forecasting, antibiotic kinetics, stress amelioration, embryo biotechnology, microbial toxins, tissue repair etc. The college has an active animal health and production research program with a candid orientation towards upliftment of socio-economic status of the people of Uttarakhand state in particular and national perspective in mind through development of livestock sector. Resource generation has been given due emphasis. Large numbers of research projects were submitted to outside agencies for funding. Many of which have already been sanctioned and others are in pipeline. Currently the College has 45 research projects worth Rs 9.00 crores in operation with financial assistance from ICAR, UPCAR, DST, DBT, etc. and 28 projects are under consideration for financial sanction by the outside agencies

The salient research achievements of the college are:

1. Isolation and purification of Salmonella cytotoxins and enterotoxins.
2. Development of toxoid vaccine against Salmonella.
3. Identification and validation of brain centers responsible for rumeno-reticular movements.
4. Identification and scientific validation of medicinal herbs for alleviation of stress and for the treatment of various animal diseases such as wound healing and fracture repair.
5. Identification of accupoints for analgesia and anaesthesia.
6. Standardization of techniques for production, purification and characterization of antisera against reproductive hormones.
7. Development of strip-test kit for detection of pregnancy as early as 18 days after conception.
8. Development of rapid strip-test for the detection of urea adulteration in milk.
9. Immunological effects of residual environmental pollutants like heavy metals and commonly used insecticides/weedicides.
10. Development of cost effective immunomodulators.
11. Development of methods for detecting toxic residues in animal products obtained from different sources.
12. Pharmacokinetic studies on antibiotics such as gentamicin, erythromycin, sulphadimidine, sulphamethoxypyridazine.

13. Genetic improvement in cattle through embryo transfer technology.
14. Surveillance and monitoring of important animal diseases.

Extension

The college is fully committed to its responsibility of the development and prompt and effective transfer of cost-effective and eco-friendly technologies to the farmers on various aspects of animal health and production. The teaching and research activities are directed towards the generation of skilled manpower, modernization of old as well as generation and validation of new technologies. Scientist-farmer-industrialist interactions are encouraged for need-based orientation of academic and research priorities. With a view to generate awareness among the rural youth and women, various training programs on poultry keeping, dairy farming, pig keeping etc. are regularly arranged. The rural masses are encouraged for participation in developmental activities and creating opportunities for income generation.

Public Health has always been an abiding concern. Besides educating people about various diseases transmissible from animals to human beings and resorting to various methods of prevention, a unique series of training butchers and slaughter house workers has been started for the production of wholesome and hygienic meat for consumers.

Since women in hills play a pivotal role in livestock farming and family management, training programs of short duration are organized to educate them on general principles of sanitation, importance of zoonotic diseases, hygienic measures and preventive aspects. Since inception, the faculty members of college have taken keen interest in solving field problems of livestock owners and have generated seventy-five sustainable technologies for the benefit of livestock owners, field veterinarians and scientists. These technologies are being disseminated to the end users.

Following the concept of "Gramabhyudayah Deshabhyudayah", the College has always been sensitive to rural upliftment. Under village adoption scheme, villages have been adopted where team of experts regularly visits for transferring new technologies through personal contacts, demonstrations, group discussions, meetings etc. Sick animals referred by field veterinarians and disease outbreaks reported from different areas are promptly attended. Mobile Veterinary Clinic has been pressed into action to provide diagnostic, therapeutic and consultancy services. Animal welfare camps, kisan gosthies and field demonstrations are organized in the rural areas of various districts to render the veterinary services and expert advice at the farmers' doorsteps. The literature on modern practices of animal husbandry development in the form of pamphlets and leaflets, are distributed. Radio and TV talks on animal related problems are also relayed for livestock owners. Redressal of animal related problems is also sought through personal contacts, letters and telephonic conversations. The College Clinic operates round-the-clock to take care of emergency and serious cases. The college has recently conducted three months training for pharmacist employed by Government of Uttarakhand to educate and provide scientific knowledge of the various subjects of veterinary sciences in four batches of 40 participants each.

Extra co-curricular setup:

Protection of students' interest has received utmost attention. Besides intensive professional training, ample opportunities are provided for co-curricular and extra-curricular activities. Co-curricular and extra curricular activities are regularly organized by 12 activity groups and through Veterinary Society for overall personality development of the graduating veterinarians. Extramural

lecture series has been introduced for all round personality development of our students. Spirit of competitiveness among UG & PG veterinary students is infused through various professional and activity groups like Personality development; Career opportunities; Literary; Cultural; Alumni; Games & sports and Prevention of cruelty to animals group. All India Competitions in the events of badminton, table-tennis, and professional quiz are annually organized.

Collaboration with other Organizations & Institutes

In its early years, the college had **a close** collaboration with the University of Illinois and a large number of faculty members obtained their higher degrees or training from University of Illinois.

There are several institutes/organizations and universities within the country working on Veterinary and Animal Sciences which are seeking collaboration with the college of Veterinary and Animal Sciences, Pantnagar for joint research projects as well as for joint Ph.D. programmes.

Future Plans

The prospective plan of the University and for College for Next 20 years has been prepared and documented in "VISION 2020". This document has identified the issues and strategies for development of Veterinary education and research in the University as well as the direction in which the future developments in Animal Husbandry should be channelized.

The university has got the responsibility for the development of Animal Husbandry in Uttarakhand which has a completely different agro-ecosystem and practices. In order to cater to the needs of the new state, necessary modification in the curriculum will be made and some new courses would also be introduced.

In view of many radical changes likely to take place in Animal Husbandry during next 20 years, some major changes/overhaul in the progamme would be necessary. More areas of specialization are likely to be created through research dynamics and new academic programmes. The necessity of making needful modifications in the undergraduate courses and starting specialized courses, which may be helpful for students in post-education settlement through self employment, will be realized more in the next two decades.

Changes in the undergraduate education in Veterinary and Animal Sciences will also be necessary so that the graduates must be prepared to take up new challenges of the 21st century in the livestock production, animal products processing, marketing and export-import business.

The infrastructure in terms of lecture rooms, laboratories, instructional equipment etc. which was created 40-45 years ago can not meet the aspirations of the new generation nor can it match the new curricula in Veterinary and Animal Sciences. Hence substitution of new equipment for the old ones, development of smart class rooms and renovation of laboratories are priority areas of new century.

DEPARTMENT OF VETERINARY BIOCHEMISTRY: A PROFILE

INCEPTION

The department of Veterinary Biochemistry was established in the year 1998, with the aim to equip the students with newer biosciences and advanced technologies. The department is facilitated by the function of Centre of Animal Biotechnology as its core unit. Since inception the department owes pride in terms of academic and research achievements at national and international level.

MANDATE

The mandate of department mainly comprise the production of quality human resource, technically sound enough to take up and shape the challenging tasks of society apart from strengthening the ongoing research in country and abroad, to combat the animal diseases and to aid the production of quality germplasm.

ESTABLISHMENT

As per the recommendations of Veterinary Council of India, VCI the number of posts sanctioned for the department to carry out the teaching research and extension activities and personnel manning them are:

Teaching staff

Post	In Position
Veterinary Biochemistry	
Professor (1)	Vacant
Associate Professor (1)	Dr. Tanuj Ambwani (Incharge)
Junior Research Officer (1)	Dr. Umapathi, V.
Assistant Professor (2)	Dr. Sudhir Kumar
Dr. Shingini Sharma	
Animal Biotechnology	
Assistant Professor (2)	Dr. Mumtesh Kumar
	Vacant

Non -Teaching Staff

Post	In Position
Lab. Technician (2)	Mr. A.B. Sati
	Mr. Balwant
Lab. Assistant (1)	Mr. P.K. Chakrovarti
Junior Assistant (1)	Mr. Mahesh Prasad
Lab Attendant (1)	Mr. Ram Nihora

Infrastructure

Besides a dedicated team of highly motivated and qualified manpower, the Department has adequate infrastructure viz. space, water, power back-up, air-conditioning, computers, audiovisual aids etc., and equipment/ instrument facilities for imparting and undertaking quality education and research. A specialized containment facility is also available for research on contagious and hazardous organisms. A laboratory animal unit provides experimental animals to all the researchers. In addition, Instructional Dairy farm (IDF) and Instructional poultry farm (IPF) of the university provides unique research opportunities and specific facilities.

Specialized Laboratories

The laboratory facilities and equipment present in Animal Biotechnology Centre is utilized for carrying out research on vaccines and diagnostics against livestock and poultry diseases. The department is well equipped with Inverted microscope, CO_2 incubators, Refrigerated Centrifuge, Spectrophotometer (UV-Vis), Laminar flow hoods, Deep freezers, Thermal cycler, FPLC, Incubator Shaker, ELISA reader, Swirling Water Bath, Gel Documentation System, PCR work station and Refrigerators. Dr. T. Ambwani and Dr. V. Umapathi are the in-charge and co-in charge of the facilities respectively.

1. **Hybridoma Lab** :- Monoclonal antibodies development against IBD virus and other antigens
2. **Genetic Engineering Lab :-** Recombinant DNA vaccine of Haemorrhagic Septicaemia and virulence gene analysis of *Salmonella*
3. **Infectious Material/ Pathogens Handling Lab:** To handle infection materials and for isolation and identification of pathogens
4. **Immunodiagnostic Laboratory:** To develop the diagnostic kits based on immunological tests for the early and efficient detection of animal diseases.

DEPARTMENTAL ACTIVITIES

Teaching

Under- Graduate Programme

VBC 111	General Veterinary Biochemistry	3(2-1-1)
VBC 121	Physiological Chemistry	3(2-1-1)
VBC 122	Introduction to Molecular Biology and Biotechnology	2(1-1-1)
VBC 411	Clinical Biochemistry	2(1-0-1)

Sl. No.	Degree offered	Subject
1.	M. V. Sc.	1. Animal Biotechnology 2. Molecular Biology & Biotechnology* 3. Veterinary Biochemistry
2.	Ph. D.	1. Veterinary Biochemistry 2. Molecular Biology & Biotechnology*

Post-Graduate Programmes

*In Collaboration with the College of Basic Sciences and Humanities, GBPUA&T, Pantnagar

No. P. G. Courses offered : Veterinary Biochemistry (M. V. Sc. & Ph. D.) - 18
: Animal Biotechnology (M. V. Sc.) - 19

Number of PG Students completed/ pursuing

Programme	Completed	Pursuing
Ph. D. (Veterinary Biochemistry)	01	01
Ph. D. (Molecular Biology &Biotechnology)	02	-
M.V.Sc. (Veterinary Biochemistry)	07	02
M.V.Sc. (Animal Biotechnology)	14	07
M.V.Sc. (Molecular Biology &Biotechnology)	02	05
M.Sc. (Molecular Biology &Biotechnology)	04	Nil
Total	**30**	**15**

Laboratory/ Training Manuals Published

- Lakhchaura, B. D., Tanuj Ambwani and Umapathi, V. (2000). 'A laboratory manual for General Veterinary Biochemistry (VBC-111)'. Pub.: College of Veterinary Sciences, G. B. Pant University of Agriculture & Technology, Pantnagar, India.
- Lakhchaura, B. D., Tanuj Ambwani and Umapathi, V. (2000). 'A laboratory manual for Molecular Biology and Biotechnology (VBC – 122)'. Pub.: College of Veterinary Sciences, G. B. Pant University of Agriculture & Technology, Pantnagar, India.
- Lakhchaura, B. D., Tanuj Ambwani and Umapathi, V. (2000). 'A laboratory manual for Physiological Chemistry (VBC – 121)'. Pub.: College of Veterinary Sciences, G. B. Pant University of Agriculture & Technology, Pantnagar, India.
- Umapathi, V., Tanuj Ambwani and Lakhchaura, B. D. (2002). 'A laboratory manual for Clinical Biochemistry (VBC-411)'. Pub.: College of Veterinary Sciences, G. B. Pant University of Agriculture & Technology, Pantnagar, India.
- Tanuj Ambwani, Umapathi, V., Sameer Shrivastava, Mumtesh Saxena, Shingini Sharma, Sudhir Kumar, (2007). A Training Manual for Immunological and Animal Cell Culture Technology, College of veterinary & Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar,

Research

Research Projects Completed

Sl. No.	Title of the project	Name of PI/Co-PI	Funding agency
1.	National fellow project on production of monoclonal antibodies against reproductive hormones	Dr. B. D. Lakhchaura	ICAR
2.	Refinement and Geld testing a bovine pregnancy procedure developed at Pantnagar	Dr. B.D. Lakhcharura Dr. Anil Kumar	DBT

3.	Development of a confirmatory test for pregnancy in cattle and buffalo.	Dr. B.D. Lakhchaura Dr. U.K. Atheya Dr. Umapathi, V.	UPCAR-DASP
4.	Antigenic characterization of field and vaccine strain of IBD virus of poultry using monoclonal antibodies produced against antigenic variants.	Dr. Umapathi, V. Dr. T. Ambwani	DBT
5.	Development of a rapid sensitive immunological molecular diagnostic test for the detection of salmonella in foods of animal origin.	Dr.V.D. Sharma, P.I. 1 Dr.T. Ambwani, P.I. 2 Dr. S.P. Singh, P.I. 3 Dr. Rashmi Singh	DBT

RESEARCH PROJECTS IN OPERATION

Sl. No.	Title of the project	Name of PI/Co-PI	Funding Agency
1.	Assessment of *Salmonella* Status of Gangetic water between Gangotri and Varanasi.	Dr. Mumtesh Kumar	DST
2.	Characterization of Potent Protective Antigens of *Pasteurella multocida* and Development of Recombinant Vaccine against Haemorrhagic Septicaemia	Dr. V.D.P. Rao, Dr. Mumtesh Kumar Dr. Rashmi Singh	DBT
3.	Molecular Typing and Virulence Gene Analysis of Poultry Isolates of *Salmonella* in North India.	Dr. Mumtesh Kumar Dr. Avadhesh Kumar Dr. Tanuj Ambwani Dr. Rajesh Kumar	DBT

Publications (Last Five Years)

(a)	Research Articles/ Papers/ Reports	: 50
(b)	Papers presented at National/ International Seminars/ Symposiums/ Trainings	: 73
(c)	Technical/ Popular articles	: 10
(d)	Books and Manuals	: 06

Awards / Recognitions

- **Dr. B.D. Lakhchaura,** Former Professor & Head was member, Task force for Animal Biotechnology, Department of Animal Biotechnology (2001-03).
- **Dr. Mumtesh Kumar** was awarded for second best research paper at national symposium on Recent Trends in Molecular Biology" organized by UGC and ICMR in the year 2006.
- **Dr. Mumtesh Kumar, Umapathi, V. and Ambwani, T**. were awarded for best research paper at national symposium by LPM in the year 2007
- **Dr. Mumtesh Kumar and Umapathi, V**. were awarded for best research paper at national symposium silver Jubilee Convention by ISVM in the year 2007.
- **Dr. Tanuj Ambwani** was awarded 'Prakash Best Poster' and 'Rajpal Best Paper' at 4th convention od Soceity of Immunology and Immunopathology (SIIP) held at Chennai, 2007

Research Achievements

- Development of immunoassay methods for steroid hormones e.g. testosterone, progesterone & estradiol.
- Modern molecular probes and immunodiagnostic techniques like ELISA, FAT, immunoblot have been adapted for detection of *Salmonella*, in animal tissues & products.
- First step in development of recombinant vaccine against bovine herpes virus-1 and H.S. and construction of an expression vector.
- Isolation and molecular characterization of *Salmonella* from Gangetic water.
- Cloning and sequencing of 'omps' genes of *Pasteurella multocida*.
- Pathological and Molecular characterization of IBDV field isolates indicated very virulent nature of Indian field isolates.
- Antigenic characterization of field isolates and vaccine strains using polyclonal and monoclonal antibodies indicated close relationship among field viruses whereas considerable difference between field and vaccine viruses.
- A Chick embryo fibroblast passaged IBDV field isolate showed attenuated nature in pathological and molecular studies. The isolate can be considered as a potential candidate for vaccine development against IBD in India
- Molecular characterization and Multi-Drug Analysis of *Salmonella* isolates.
- PCR based rapid detection of *Salmonella* and Pasteurella in food and clinical samples.
- Molecular analysis of virulence gene of *Salmonella*.

OTHER ACTIVITIES

Linkages

The Department is actively linked with Indian Veterinary Research Institute Izatnagar and Mukteswar campus, in addition to other departments of the university viz., Department of Veterinary Medicine, Surgery and Radiology, Pharmacology and Toxicology, Anatomy, Livestock Production and management, Extension Education, Physiology, Microbiology, Public Health, Animal Reproduction Gynecology & Obstetrics, Genetics and Animal Breeding, Animal Nutrition, and Pathology and Molecular Biology & Genetic Engineering.

Training/ Conference Organized

State Biotechnology Programme Government of Uttarakhand Sponsored Short Term Training *(22-28 January 2007)* on "Immunological and Animal Cell Culture Technology". Course coordinators were Dr. Tanuj Ambwani and Dr. V. Umapathi.

The department also organized A short term training course on 'Diagnostic ELISA', from Nov. 26 to Dec. 10, 2007.

FUTURE PLANS

The Department has embarked upon strengthening the teaching with advanced technologies both at UG as well as PG level with particular reference to Uttarakhand state

DEPARTMENT OF VETERINARY MICROBIOLOGY: A PROFILE

INCEPTION

The Department of Veterinary Microbiology has been established in the faculty since its inception in 1960. The department is continuously providing skillful teaching to undergraduate and postgraduate students, which has been possible only due to exhaustive research on Salmonellosis and *E.coli* infections and their enterotoxins and cytotoxins, mastitis, staphylococcal infections and food intoxications; New Castle Disease Pasturellosis, Parvovirus Infections, pox infections of domestic animals and birds, Marek's disease, infectious bursal disease and Inclusion body hepatitis/hydro pericardium syndrome of poultry. The department has always undertaken problem oriented research work and has contributed significantly in developing teaching models, and immunodiagnostics and vaccines. It is charged with the responsibility of teaching, research and extension in the areas of veterinary bacteriology, virology, mycology and immunology.

MANDATE

The mandate of the department is to carry out UG/PG teaching and research to support teaching and research in Veterinary Microbiology and Immunology. In order to strengthen the teaching with advanced technologies for meeting emerging challenges in infectious diseases of livestock and poultry and to provide better health coverage and animal welfare

MAN POWER

As per the recommendations of VCI the number of posts sanctioned for the department to carry out teaching, research and extension activities and personnel manning them are:

Teaching

Post	**In position**
Professor (1)	Dr. V. D. P. Rao (Head)
	Dr. R. S. Gupta
	Dr. Rajesh Chandra
Associate Professor (2)	Vacant
Assistant Professor (2)	Promoted as Professors
Junior Research Officer	Dr Rashmi Singh

Non-Teaching

Research Assistant	Mr. J.B. Sharma
Sr. Lab. Assistant	Mr. Bishan Singh Mehra
Lab. Assistant	Mr. Phoolbas
Junior Assistant	Mr. Mohd. Amin

ACTIVITIES

Teaching

Under Graduate: Veterinary Microbiology and Immunology has become more structured after introduction of VCI recommendations, with a total about 256 lectures and 448 practical/ demonstrations over a period of three years. Practical session included virology, bacteriology, immunology, animal cell culture, diagnosis of microbiological samples from clinical cases. Department is offering six courses as per the recommendation of VCI. For easy understanding, complete coverage of each experiment and for benefit of students following manuals have been developed so that it can be used in succeeding years:

1. Laboratory Manual of Veterinary Virology
2. Laboratory Manual of General Veterinary Microbiology
3. Laboratory Manual of Veterinary Immunology & Serology
4. Laboratory Manual of Veterinary Bacteriology & Mycology

Post Graduate: This department is one of the few departments of the college which started post graduate programme as early as in 1964 So, far as many as 105 and 25 students have earned their M.V.Sc. and Ph.D. degree in Veterinary Microbiology & Immunology and are occupying distinguished positions in different teaching, research and industrial organizations in India and abroad. Following courses are being offered to M. V. Sc./Ph. D. students:

P.G. Programmes : **M.V.Sc. (Veterinary Microbiology & Immunology)**

Ph. D. (Veterinary Microbiology & Immunology)

No. of P.G. Courses : **18**

The department continues to distinguish itself in research areas such as immunodiagnostics, epidemiology and vaccines for bacterial and viral diseases of livestock and poultry.

Number of Theses submitted in the Department of Veterinary Microbiology

M.V.Sc. : **86**

Ph. D. : **17**

Research

The major thrust areas of research have been animal pox, enterobacteria and their toxins, reservoir of zoonotic diseases, mastitis, antimicrobial property of indigenous plants and avian diseases of viral origin. Department has done significant research on Salmonellae and developed a vaccine for its control in poultry. This vaccine has been granted patent in USA Germany and also in India. Apart from this other Vaccines developed by scientists of this Department are sheep pox vaccine, infectious bursal disease vaccine, New castle disease vaccine and an autogenous vaccine against hydro pericardium syndrome virus. Department has also developed a rapid test to detect urea adulteration in milk.

A. Research Projects

Department has successfully handled 15 externally funded research projects and 3 research

projects (one supported by ICAR and 2 by Department of Biotechnology) are currently running in the Department.

1. Research projects completed

Sl. No.	Name of the Project	Principal Investigator	Funding Agency
1.	Investigation on Marek's Disease in poultry with special reference to its epidemiology, diagnosis and control	Dr I. P. Singh Dr S. K. Garg	ICAR
2.	Rservoirs of zoonotic diseases in Uttar Prdesh	Dr M. S. Sethi Dr V. B. Singh	PL-480
3.	Immunologic characterization of buffalo pox virus and immunity in buffalo pox	Dr I. P. Singh	ICAR
4.	Development of suitable vaccine for sheep pox and characterization of its immune response in sheep.	Dr I. P. Singh	ICAR
5.	Studies on incidence of drug resistance enterobacteria	Dr D. S. Misra	UGC
6.	Anti-enterotoxigenic property of Garlic (*Allium sativum. L.)*	Dr V. D. Sharma	BARC
7.	Studies on (a) enterotoxigenicity of Salmonella and *E. coli* with particular reference to their pathogenesis and (b) the biochemical and immunological characterization of their enterotoxins	Dr I. P. Singh	ICAR
8.	Studie son plasmid mediated drug resistance of bacteria isolated from clinical and non-clinical animal sources	Dr V. D. Sharma	ICAR
9.	Enterotoxigenic Salmonella in foods of animal origin and characterization of their enterotoxins.	Dr V. D. Sharma	ICAR (National Fellow Project)
10.	Studies on Infectious bursal disease of poultry:	Dr V. D. P. Rao	UPCAR
11.	Development of suitable, potent and cost effective vaccine against Newcastle Disease virus	Dr S. K. Garg	ICAR
12.	Development of Diagnostic kit and vaccine for Capripox infection in sheep and goats.	Dr V. D. P. Rao	UP Govt.
13.	Isolation and characterization of etiological agent	Dr Rajesh Chandra	ICAR

	of Hydropericardium syndrome and its control by a suitable vaccine.		
14.	Veterinary diagnostics for prevalent and emerging diseases of canine.	Dr S. K. Garg Dr Rajesh Chandra	NATP, ICAR
15.	Animal Health Information system through disease monitoring and surveillance	Dr V. D. P. Rao	NATP, ICAR

2. Research Projects in operation

Sl. No.	Name of the Project	Principal Investigator	Funding Agency
1.	Development and evaluation of cell culture adapted vaccine against fowl pox	Dr. V.D.P. Rao	ICAR
2.	Characterization of potent protective antigens of Pasteurella multocida and development of recombinant vaccine against Haemorrhagic septicaemia	Dr. V.D.P. Rao	DBT
3.	Serological and molecular characterization of etiological agents of hydro pericardium syndrome with special reference to development of suitable cell culture vaccine	Dr. Rajesh Chandra	DBT

B. Research Publications:

The department believes in dissemination of scientific knowledge accrued through publication which is evident from the fact that about 750 research papers have been contributed by the department Of these, 75 research papers have been published in International Journals, 325 in Indian Journals, 250 papers presented at various conferences/symposia. In addition 30 review articles, 5 research bulletins, 120 popular articles have been published by the academic staff of the department in national and International journals. Faculty members of the department have also written/ contributed in writing two books namely " i'kq fpfdRlk thok.kq foKku"ys [kd& Dr. V.D. Sharma and "Diseases of Poultry and their Control" by Dr. Rajesh Chandra, Dr. V.D.P. Rao and others. Department has also published 4 laboratory manuals in the field of bacteriology, virology, Immunology and mycology.

C. Salient research achievements:

Salmonellosis

- Out of 7.725 samples (fecal/intestinal contents/visceral organs) from different animals (domestic/wild/zoo/house pets/avifauna) examined, 361 (4.54%) yielded Salmonella belonging to 25 serovars.
- Out of 4.155 samples of commercial foods of animal origin (kawab, salami, meat pie, hamburger, bacon, chocolate, frozen chicken, frozen fish etc.) examined, 182 (4.38%) were found contaminated with Salmonella belonging to 24 serovars. 93% and 65.5% of Salmonella isolates from foods were found cytotoxigenic and enterotoxigenic, respectively.
- An enterotoxin and four cytotoxins of Salmonella have been purified to homogeneity and

characterized (physicochemically, biologically and antigenically) for the first time.

- A novel, safe and potent formalized toxoid vaccine has been developed against salmonellosis. It afforded 100% protection in mice and poultry, checked shedding of challenge Salmonella and induced passive immunity in chicls/

Brucellosis

- Of the 4.144 human and 10.546 animal serum samples examined, 19 (0.45%) and 377 (3.56%), respectively, were found positive for Brucella agglutinins.

Coxiellosis

- Of the 16.334 samples (serum and milk) screened for Coxiella burnetti agglutinins, 2,166 (13.24%) were found to be positive.

Antimicrobial activity of indigenous herbs

- Essential oils of garlic, Ocium spp. and Eucalyptus spp. markedly inhibited the growth of pathogenic bacteria and fungi.
- Garlic extracts cured candidiasis in poultry and ringworm in human beings, dogs and horses.

Pox

- Pathogenesis of buffalo pox virus has been studied in detail in rabbit and buffaloes.
- A live attenuated cell culture vaccine against sheep pox was developed. It was found to be safe and 100% effective in a field trial conducted at Jorbid Sheep Farm, Bikaner.
- Immunodiagnostics for capripox infection in sheep and goat
- Development and Evaluation of cell culture vaccine against fowl pox

Infectious bursal disease

- Twenty outbreaks of IBD were recorded during the year 1994-95 in the nearby area. The virus was isolated from different outbreaks in and around Pantnagar in chicken embryo fibroblast cell culture and on BGM70 cell line. An attenuated vaccine using local isolate has been developed.

Hydropericardium syndrome

- An autogenous vaccine was developed using livers from naturally affected birds. The vaccine was used in more than 200,000 broilers in Tarai of U.P. It has significantly contributed in controlling the disease in Tarai. A fowl adenovirus (FAV-4) has been isolated in chicken embryonic liver cell culture.
- Serological and molecular characterization of field isolates, from different parts of country, of Fowladenovirus associated with IBH-HPS in domestic fowl.

Canine Parvo virus

- Immunodiagnostics for canine parvovirus

Strip test for diagnosis of urea adulteration in milk

Haemorrhagic septicemia

- Characterization of immunogenic OMPs of *Pasteurella multocida* and development of recombinant vaccine using genes coding for these proteins.

Awards and honours: Recognition of the services of the academic staff of the Department is evident from following awards and honours conferred on them:

Award (s)	Name
Dr K. S. Nair Memorial award	Dr S. K. Garg
Life time achievement award for poultry scientists	Dr V. D. Sharma
National fellow award	
Fellow of IAAVR	
Shyam Singh Balamati Memorial award	
Fellow of NAVS	
Dr C. M. Singh award	
Dr K. S. Nair Memorial award	Dr V. D. P. Rao
ICAR Best Teacher Award	
Fellow of NAVS	
INSA visiting fellowship	Dr R. S. Gupta
Commcn Wealth Academic Staff Fellowship	Dr Rajesh Chandra
Best Research Worker award	
Fellow of NAVS	
Best Teacher Award	

Extension:

To provide Microbiological diagnosis of diseases of livestock and poultry to farmers. In addition to this scientists of the department also give expert lectures in the training programmes organized for benefit of farmers, Vets and Paravets.

FACILITIES

Infrastructure and Instruments/Equipments

Department has separate undergraduate, postgraduate and research laboratories viz., Virology Laboratory, Microbial Toxins Laboratory, Enterobacteria Laboratory, Immunology Laboratory and Diagnostic Microbiology Laboratory as well as seminar room and department library, adequate office space for staff members and sufficient animal houses for poultry and small animals. The department has SDS-PAGE assembly with western blot apparatus, DNA gel electrophoresis assembly with southern blotting apparatus, transilluminator, Deep freezers, ultrafreezers, Laminar

flow systems, Inverted phase contrast Microscope, Fluorescent Microscope, gradient thermal cycler, refrigerated centrifuges, sinicator, ELISA reader, lyophilizer, electronic balance, CO_2 incubator, normal incubators, egg incubator, digital pH meter, autoclaves, hot air ovens, dryers water distillation plants, general laboratory microscopes, shaker incubator in addition to all minor equipments which are required for UG/PG teaching and research.

SUMMER SCHOOL/SYMPOSIA ORGANIZED:

The department had contributed to advancement of Veterinary Microbiology and Immunology in form of organizing various summer schools and symposia; details are given below:

1. Summer School on Animal Pox Virus: June, 1972
2. Summer School on Microbial enterotoxins and their impact on animal and human health: June, 1982
3. Summer School on Recent advances in meat microbiology with special reference to meat-borne infections and intoxications: June, 1986
4. Summer School on Exploitation of biomolecules of pathogens for immunodiagnosis and immunoprophylaxis: June, 1997
5. National symposium on Trends in Vaccinology for Animal Diseases: October, 2000.

FUTURE PLANS

The department has embarked upon strengthening the teaching with advanced technologies both at UG as well as PG level. For this some area have been identified which needs to be taken up on priority basis:

Teaching:

1. Development of new courses for M. V. Sc. and Ph. D. teaching and pertinent text books on various subjects for teaching and laboratory manuals for PG teaching.
2. Developments of bacterial and viral models for better understanding.
3. Development of multimedia/CD's/simulated programmes on animal cell culture preparation, various techniques of animal/egg inoculation and other various microbiology and immunology experiments

Research

Future research priorities of the department are:

1. Development of rapid and cheap diagnostic kits and newer generation cost effective vaccines for various infectious of livestock and poultry prevalent in India with particular emphasis on Uttarakhand State.

Extension

1. Training technical staff diagnosis of infectious diseases of livestock and poultry
2. Preparation of leaflets/instruction material for protection of animals against infectious diseases.
3. Provide technical know how to farmers for protection of livestock and poultry from infectious agents.

XIV Annual convention of Indian Society for Veterinary Immunology & Biotechnology. (ISVIB) and National Symposium on Recent Trends in Immuno-Biotechnology based Biologicals and their Commercialization

February 27-29 2008

TECHNICAL PROGRAMME

Date	Time		Programme
	From	To	
27-02-2008	09.00 AM	11.30 AM	Registration
	11.30 AM	12.30 AM	Inaugural Session (I)
	12.30 AM	01.00 PM	Inaugural Tea
	01.00 PM	02.00 PM	Lunch Break
	02.00 PM	03.30 PM	Session II
	03.30 PM	03.45 PM	Tea Break
	03.45 PM	05.30 PM	Session III
28-02-2008	09.00 AM	11.00 AM	Session IV
	11.00 AM	11.15 AM	Tea Break
	11.15 AM	01.00 PM	Session V
	01.00 PM	02.00 PM	Lunch Break
	02.00 PM	03.30 PM	Session VI
	03.30 PM	03.45 PM	Tea Break
	03.45 PM	05.00 PM	Session VII
	05.00 PM	06.00 PM	General Body Meeting
29-02-2008	09.00 AM	11.00 AM	Session VIII
	11.00 AM	11.15 AM	Tea Break
	11.15 AM	01.00 PM	Plenary Session (IX)
	01.00 PM	02.00 PM	Lunch Break
	02.00 PM	03.00 PM	Valedictory Session (X)

Session I	Inaugural Session
Session II	Dr. P. Richard Masillamony oration and Key Note Address
Session III	Conventional and Novel Vaccine Approaches
Session IV	Design & Development of Molecular &/or Immuno diagnostics
Session V	Role of Genomics & Proteomics in development of Biologicals
Session VI	Bioinformatics: Applications for development of Biologicals
Session VII	IPR and commercialization issues for Biologicals
Session VIII	Production Biotechnology and their commercial aspects
Session IX	Plenary Session
Session X	Valedictory Function

SESSION - I

Inaugural Session

SESSION - II

Dr. P. Richard Masillamony Oration and Key Note Address

II.1 Dr. P. Richard Masillamony Oration
Dr. M.S. Oberoi

II.2 Key Note Address
Dr. George John

SESSION - III

Conventional and Novel Vaccine Approaches

Chairman : **Dr. S.K. Garg,**
Co-Chairman : **Dr. Rajesh Chandra**
Rapporteur : **Dr. Rajesh Kumar**

LEAD PAPERS

III.1 Quality Control and Bio-Safety Issues Related To Veterinary Viral Vaccine Development
B.Pattnaik and Aniket Sanyal

III.2 RNA Replicon-based Self-Replicating DNA Vaccines
Praveen K. Gupta

III.3 TLR Ligands - A class of novel Vaccine Adjuvants
V. Ramaswamy

III.4 Cowpathy for Immunomodulation of Vaccine Response
R.S. Chauhan

III.5 Recent Developments in the Animal Virus Vaccines
Prof (Dr) M. P. Yadav and Dr R.K. Singh

ABSTRACTS

3.01 Study on outbreaks of sheep pox in Karnataka, India from 2001 to 2006
Raveendra Hegde, Amitha R. Gomes, H.K. Muniyellappa, S. M.Byregowda, P. Giridharand C. Renukaprasad

3.02 Preparation and testing of inactivated oil emulsion Infectious Bursal Disease virus vaccine of Vero cell culture origin
A. Thangavelu, K.S. Palaniswami and V. Purushothaman

3.03 A test for *in vitro* potency determination of Haemorrhagic Septicaemia vaccines
Anil Kumar Mishra and Mayank Rawat

3.04 Study of protein profile of Jaipur strain of Sheeppox virus using SDS-page
Shingini Sharma and Rajesh Chandra

3.05 Preparation and characterization of chitosan coated PLG nanoparticles for delivery of DNA vaccines
P., Arvind K., Ramya, K. and Ram G.C. Chaudhari U.K., Dandapat S., Chaudhury

3.06 Study on epidemiology of Peste des petits ruminants infection in Karnataka state, India
Raveendra Hegde, Amitha R. Gomes, H. K. Muniyellappa, S. M. Byregowda, P. Giridhar and C. Renukaprasad

3.07 Evaluation of Immune Status of the Cattle Vaccinated against Brucellosis with RB 51 Strain
Khalid Ali, M. Nadaf & Y. Hari Babu

3.08 Management of EDS-76 virus infection in Japanese quail through Vaccination
B.B. Dash, Susitha,K., S. D. Singh, S. K.Gupta and Visnuvinayagam, S.

3.09 Conventional and novel vaccine approaches
B.K.Barun

3.10 Novel TB vaccines
B.K.Barun

3.11 Thermostable PPR Virus- Role of Deuterium in conferring heat resistance
Arnab Sen, V. Balamurugan, Vinita Yadav, Riyesh T, Vandna Bhanot, and R.K. Singh Singh

QUALITY CONTROL AND BIO-SAFETY ISSUES RELATED TO VETERINARY VIRAL VACCINE DEVELOPMENT

B.Pattnaik and Aniket Sanyal*
Project Director, •
Project Directorate on Foot and Mouth Disease,
Indian Veterinary Research Institute Campus,
Mukteshwar – 263 138, Uttarakhand, India
*Sr. Scientist

Vaccination is a single most important measure to contain and in limited extent to treat infectious diseases. Vaccine is a final product obtained after employing complex manufacturing and production techniques. Growth of virus in cell culture, which utilizes substances of animal origin like foetal calf serum, trypsin etc. may lead to accidental introduction of contaminant. Each ingredient utilized during vaccine production should be free of contaminants and inappropriate agents as generally the final product is not sterilizable. Sometimes release of pathogenic infectious agent is a danger to animal and also to human population if it is zoonotic in nature. Any vaccine manufacturing and production unit need to think about some of the critical points mainly related to biosafety practice (possible contamination, cross-contamination and accidental exposure of the agent to workforce and surroundings).

Quality is "the totality of features and characteristics of a product or service that bears on its ability to satisfy stated or implied needs". Safety and efficacy are also pertinent to quality of medicinal product such as vaccine. In addition, simplicity, compatibility and cost are the other components of quality. If the vaccine is manufactured and designed properly then only it can meet the required standard of quality.

The final product (vaccine) must be pure, safe, efficacious and potent. Purity means vaccine is free of extraneous microbes or extraneous materials which adversely affect safety, efficacy or potency. Safety is freedom from properties causing undue local or systemic reactions. Efficacy for biological material is defined as the specific ability or capacity of the biological product to effect a result for which it is offered when used under the condition recommended by the manufacturer. Efficacy of a vaccine is the ability to protect vaccinate from the disease. Potency is a relative strength of a biological product as determined by test methods or procedures. in standard requirements or in the approved outline of production for such product (Levings *et al.*, 1993). Quantity of specific antigenic material present and its comparison to the vaccine dose shown efficacious is evaluated by assays involved in potency testing.

Sampling during production (raw materials, bulk, packaging materials, intermediate and final product), noting down the specification related to the product and tests carried out encompass quality control. Quality control certifies authenticity of organization, documentation, vaccine release procedure and circulation of vaccine for sale/supply only after it is found satisfactory.

Quality control utilizes controlled, standardized methodologies to test ingredients at various stages of the production process. Information such as formulas for calculations and test procedures is usually defined in pharmacopoeias. Quality control should be well informed prior to conducting its mandate by production unit as testing of samples is carried out at various stages of production. Change in production like scaling up and changes in equipment, methods of mixing and ingredi-

ents may affect requirement of antibiotics and pHs and thus have impact on quality control system.

Quality assurance is "all those activities planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality".

Role/requirements of Quality control

Quality control governs various issues related to vaccine production. Activities performed by Quality control unit and requirement for their executions are pointed out in the succeeding text...

- Quality control unit undertake sampling and testing of ingredients, components, reagents and raw starting materials etc. Sampling is done by qualified personnel and requires adequate facilities, trained staff and knowledge of approved validated test procedures for inspection and testing
- It also look for glitches, conduct regular re-assays of reagents and help in testing manufacturing discrepancies
- Sometimes numerous test methodologies are available, in such case it makes certain that the procedures written in the product summary are the same to those which are conducted
- It testify the list provided to government authorities on raw materials and components are alike to those utilized in the method
- It confirm that the regulatory authorities are informed about any changes in the procedures or ingredients prior to its implementation
- It makes sure about that the records are furnished in the manual or recording instrument or not? Generally those are kept in the form of bench records providing type and test results, formulae, citation of established test methodology etc. as proof of genuineness of actions. Quality control also confirms that the records contain results of testing measured against specifications. Information pertaining to quality control also offered as raw material assays, sampling logs, stability programme data, certificates of analysis, notebooks of lab instrument calibration and animal handling unit etc.
- It make sure that the final product (vaccine) include active ingredient which meet the terms with qualitative and quantitative composition endorsed in the market
- It verify purity of final product (vaccine), its enclosure in apposite container with apt labelling
- It certify release and sale/supply of final product (vaccine)
- It ensures that samples (starting material and final product) are properly stored in adequate quantities for future examination

The quality control unit works closely with Research and Development section, Regulatory affairs office, Material and Management departments, Engineering and maintenance section and Office of Quality assurance. Each one has unique role to play in assisting the quality control unit. Wherever possible quality control unit authorities are also involved in carrying out the functions

along with these sections and keep themselves acquainted to any changes effected. Research and Development section may involve in checking the stability of the product, which is one of the topics of investigation among many of such issues. It monitors effect of time, preservative, heat, light, vial, stopper composition and temperature on the product. Regulatory affairs unit testifies whether the abbreviated version of expiry date, storage temperature and product ingredients are mentioned in the label or not. Keeping updated information, the office also alerts to quality control authorities on the questionable stability and status of the product. Material and Management departments may determine and inform about quantity of sample acceptable, quality level, re-assay of reagent and ingredients retained for one year or more. The departments also provide desired space to carry out sampling. It also involves in checking the certificates of analysis, tests done and results obtained and also analogous sampling and testing in different laboratory set up until the satisfactory confidence level is achieved. Engineering and maintenance section keep eye on water systems changes in service systems, existing drains, remodeling of facility etc. The section also helps in calibration of instrument. Office of Quality assurance re-examine the data generated by quality control before releasing final product (vaccine) and it supervise quality control to ensure observance to all valid standards and test procedures.

Bio-safety practices revolve around safety of personnel, environment and product. Depending on the nature of vaccine (killed or live attenuated) the precautions during vaccine production and manufacturing procedure need to be addressed. In commercial production of live vaccines using chicken egg embryo and tissue culture derived from them as well as from bovine cell culture, residual viruses may contaminate the vaccine. It is imperative to use foetal calf serum, which is free of Bovine Viral Diarrhoea Virus for vaccine production. For handling any infectious agent to produce vaccine, first step to do is to allot/identify a risk group entitled to that agent. Different agencies have divided the infectious agents into separate risk group classification by considering epidemiological aspect, infectivity of the agent, stability, ability to cause infection by various routes and susceptibility of the agent; this is called as risk assessment which is regarded as backbone of bio-safety practice. World Animal Health Organization has classified (Table 1.) infectious agents into four groups (Group 1,2,3,4 Animal Pathogens). Again, risk assessment varies while using genetically modified organisms considering hazards associated with host/recipient and directly by inserted gene. Dr Nathan Zygraich pointed out importance of recombinant DNA production of protein and hybridoma production of monoclonal antibodies in vaccine development. Advance in biotechnology lead to invention of new genetically engineered viral vaccines like i) Attenuated live vaccine by deleting specific genes or regions responsible for virulence yielding replicating non pathogenic virus (Pseudorabies) ii) Defective live vaccine by blocking replication (Feline leukemia) after early gene expression but before DNA replication iii) Chimeric live vaccines by recombinant technique immunizing against more than one virus (Capripoxvirus, Rinderpest, Lumpy skin disease) iv) Subunit vaccines using subviral components as immunogens along with adjuvants (Bluetongue) v) DNA polynucleotide vaccines using biolistic gun (gene gun) which introduce DNA directly into the muscle (Avian influenza) and vi) Polypeptide vaccines using synthetic short polypeptides of 20 amino acids as immunogens (Foot and Mouth Disease). Accordingly vaccine production unit need to use the proper containment level with availability of three microbiological safety cabinets. It is inevitable to conduct experimental and in-house trials in initial development of vaccine using animals, laboratory as well as host. Animal facility bio-safety levels are designated and those are utilized for carrying out the experiments. Primary, secondary and tertiary barriers are important to prevent the infection. Various emergencies like fire, flood, earthquake etc. are also taken into consideration before designing and constructing the building. Bio-safety officer and

Bio-safety committee along with other officials negotiate smooth functioning of the laboratory. As usual, knowledge and awareness regarding bio-safety practices help workers to achieve better results.

When an immunogenic portion on the virus or whole virus is found efficacious in initial studies, its large-scale production for vaccine preparation commences. Now amenities required for large-scale production of virus by vaccine manufacturer may be totally changed as that of the starting experiments. Taking into account nature of organism, processes involved in the manufacturing, economic aspect, technical realities and geographical situation design and operation facilities suitable for vaccine research are planned. Several things recurring as well as non recurring like construction of building, equipments needed, air handling system, functional staff, supply of water, media preparation, seed production and storage, antigen production, antigen inactivation, vaccine blending are thoroughly thought of and nuisances with possible solutions are kept in mind before actual beginning of large scale vaccine production. After production of vaccine its suitable storage till it sells in the market is also an important issue. Detailed guidelines for practicing bio-safety are found in laboratory bio-safety manual of World Health Organization and other relevant literature.

Vaccine research is a combination of basic research and progressive experimentation. Related information to some of the important viral vaccine production and challenges to it:

- Some viruses are spread rapidly, and one of them Foot and Mouth Disease (FMD) is a 'Fast Moving Disease'. Utilising FMD virus in live form is always risky as the disease is of huge economic importance and therefore stringent bio-safety measures are followed by handling live virus in high containment area. FMD is one of the OIE list 'A' disease. Dr H.K. Pradhan classified FMD virus in Risk group IV and exotic FMD viruses in Risk group V. Inactivated antigen, whose safety is not measured, is evaluated under high containment area.

- Animal trials for vaccination-challenge etc. are conducted under suitable containment facility. When challenge studies involve arthropod-borne viruses like Bluetongue etc., necessary steps are taken to avoid exposure of challenged sheep to arthropods.

- Aggregated antigens attach to the inner surface of bottle/container (Bluetongue, FMD) and hidden live virus lying in the interior may not get inactivated completely, ultimately it is a risk for vaccination. It is a simple practice in laboratories to change the container and allowing sufficient time to complete inactivation.

- The viruses originating by vaccine escape mutants, genetic drift, genetic shift, recombination, re-assortment mechanisms, represent stiff challenge in development of effective vaccine. Yearly updating the viral strain eliciting the protective immune response is practiced in development of vaccines against such viruses. For this reason, some agencies help to vaccine manufacturer by prediction of viral strains. To make the vaccine for avian influenza naturally occurring strains of which the neuraminidase type is different from the circulating field virus may be used because main protective immune response is to the haemagglutinin protein.

- The cost of production for per dose of vaccine should be less (in human it is projected as Rs. 40-50/dose) if it has to cover every animal under the umbrella of immunity. But in the present scenario research and development costs for designing modern vac-

cine is much more beyond the expectations. At times vaccine may be a failure because of many reasons. Then, vaccine manufacturer may have to bear liability expenses.

- High security laboratories conduct vaccine research on exotic or emerging diseases. Our country never had experienced avian influenza outbreaks before last few years but now it is creeping along various places and exhibiting its fangs regularly. Fortunately, there is no report of human case in these outbreaks. High Security Animal Disease Laboratory located at Bhopal serving the nation with dedication and urgency during the outbreaks by timely diagnosis and preparation of vaccine. Bird flu has potential to become pandemic. Therefore, such organisms are handled in specialised containment facilities, which are only available and entitled to High Security laboratories.
- Internationally there are 9 major and several other high security laboratories (AAHL,

Table 1. Risk groups [1]

Character/Measure	Group 1 Animal Pathogens	Group 2 Animal Pathogens	Group 3 Animal Pathogens	Group 4 Animal Pathogens
Enzootic	Yes	Yes	Yes	Yes
Exotic	No	Yes	Yes	Yes
Risk of spread	No	Low	Moderate	High
Official Control	No	Yes	Yes	Yes
Vector-borne	No	No	Yes/No	Yes/No
Economic importance	No	Limited	Severe	Extremely severe
Quarantine applied	No	No	Yes	Yes
Movement controls	No	No	No	Yes
Examples [2]	MD, FeLV, BoLV, BoPV	NDV, EEE, VEE, Orf, Influenza viruses type A,B,C,	Rabies, Japanese B encephalitis, Louping ill	Ebola, Marburg, RVF, Hantaan
Bio-safety cabinet	Open Bench/Class I	Class I	Class I,II or III	Class III, Class II with positive pressure suits

1- Risk groups may vary depending on agency and country
2- Some viruses assigned in different risk groups

Australia; NCFAD, Canada; PIADC, USA; DVIV, Denmark; FRCVDA, Germany; CVI, Netherlands, CISA, Spain; IVI, Switzerland and IAH, UK). Few of them are conducting vaccine research in defining new epitopes, investigating delivery vehicles and using plants in producing animal virus antigen. Notably, Plum Island Animal Disease Centre constructed a non-infectious FMD virus by deleting cell receptor binding sequences. Though, this virus is difficult to grow in cell culture in bulk, it is immunogenic without producing infection in animals. So, PIADC developed a new receptor on cell culture for FMD virus. A peptide vaccine is developed against canine parvovirus incorporating plant adjuvant by Danish Veterinary Research Institute for Virus Research.

It is quite difficult to talk on each aspect on quality control and bio-safety practices in such a short discussion. Both of them are complementary to each other in coming out with the standard vaccine which will not only satisfies the needs of the purchaser but also prove great savior to susceptible animal that needs it most.

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RNA REPLICON-BASED SELF-REPLICATING DNA VACCINES

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Vaccine vectors based on recombinant viruses have been used for many years to deliver target antigen, but the delivery of target antigens using this method is accompanied by unwanted side effects. First, preexisting antibodies that can neutralize the recombinant virus before it is able to deliver its payload. Second, structural proteins from the virus can dominate T- and B- cell–mediated immune responses, diverting immunity away from the target immunogen. Hence, there has been a critical need to develop vaccine vectors that are not only highly immunogenic, but also antigenically simple. The simplest of all recombinant vectors is naked plasmid DNA vaccines. Vaccination with plasmid DNA is an immunization strategy that has great potential for the development of vaccines and immune therapeutics. DNA vaccines are easy to produce, inexpensive and safe and immunogenic for several antigens. This strategy has been proved to be highly effective in mice to induce immune responses to many pathogens and model antigens, while less immunogenic in humans and animals including, nonhuman primates. Further, drawbacks of DNA vaccination include long-term uncontrolled expression of a transgene, possibility of integration into the host genome and possible induction of anti-DNA antibodies. Enhancing DNA vaccine potency remains a challenge in large animals and human. For this reason, research on DNA vaccines has moved to its second phase with the emphasis now on improving immunogenicity and efficacy. This includes: (i) improved DNA plasmids used as vectors in an attempt to enhance antigen expression by strong promoters and focus antigen targeting; (ii) better delivery systems for more efficient transfection of cells *in vivo*, including delivering a DNA vaccine together with a conventional adjuvant such as monophosphoryl lipid A (MPL); and (iii) the development of molecular adjuvants to enhance immune responses, including the co-delivery of cytokine encoding plasmid "adjuvant-plasmid" or other adjuvant molecules. Despite these modifications, the poor immunogenicity of plasmid DNA remains apparent when attempting to elicit protective immunity.

One of the possible reasons for insufficient immunogenicity of conventional DNA vaccines has been hypothesized to be low antigen production from the plasmid DNA. To increase antigen production from DNA vaccines a new strategy has been developed to express the target heterologous antigen under the control of replicase from positive-strand RNA viruses with the premise of using the ability of these viruses to produce large amounts of viral mRNA in infected cells. The replicase-based vectors of positive-strand RNA viruses are becoming more and more popular for development of antiviral and anticancer vaccines. Several features make these vectors a desirable choice for development of highly efficient and safe vaccines. These include (i) a high level of expression of encoded heterologous genes, including immunogenic genes from variety of pathogens, due to the ability of replicon RNA to amplify itself; (ii) exclusively cytoplasmic replication, which eliminates any possible complications associated with nuclear splicing and/or chromosomal integration; (iii) inability of the replicon RNA to escape from the transfected cell, thus limiting the spread of the vaccine vector in the immunized subject, which makes these vectors biologically safe. An additional beneficial feature of replicon vectors is a variety of modalities that can be used to deliver replicon RNA into cells. It can be either delivered directly as naked RNA transcribed in vitro (RNA based), packaged first into virus-like particles (VLPs) and then delivered via infection with these VLPs, or delivered in the form of plasmid DNA (self-replicating DNA vaccines), which encodes replicon cDNA placed under the control of a mammalian expression pro-

moter, allowing the production of functional replicon RNA in vivo by the cellular RNA polymerase II.

RNA replicon-based expression vectors have been developed for representatives of most positive-strand RNA virus families, namely, *Togaviridae*, *Flaviviridae* and *Picornavirus*. Several members of *Alphavirus* genus of *Togaviridae* family, including, Sindbis virus, Semliki Forest virus, Venezuelan equine encephalitis virus and *Flavivirus* genus, including, tickborne encephalitis virus, Kunjin virus, Pestivirus-BVDV and Coronavirus- HCoV have received considerable attention. However, the great majority of the data on immunogenic properties of replicon vectors in laboratory animals have been accumulated using replicons Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus.

Mechanism of RNA-replicon based DNA vaccine to produce heterologous antigens

Alphaviruses are enveloped viruses with single-stranded, positive-strand RNA genomes. The 5' end of the genome encodes the nonstructural genes, while the 3' end encodes the structural genes. The nonstructural genes are translated from the genomic RNA after its release into the cell cytoplasm. These nonstructural proteins form a replication complex that drives the production of the negative-stranded anti-genomic RNA and also transcribes the full-length positive stranded genomic RNA and a shorter subgenomic RNA. The subgenomic RNA encodes the structural genes. This arrangement allows for rapid multiplication of subgenomic mRNA in that as the full genomic RNA is replicated there are more templates for transcription through the subgenomic promoter, leading to exponential amplification of the subgenomic RNA. Alphaviral replicons are created by the replacement of the structural genes with heterologous genes, abrogating the virus' ability to replicate due to the loss of its structural genes while allowing for extremely high expression of the transgene.

All three delivery modalities were effective; however, VLP delivery was shown to be the most efficient. In comparative studies of conventional (nonreplicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally induced stronger immune responses and at significantly lower DNA concentrations than did conventional vectors. The heterologous gene expression in the conventional DNA vaccine construct is driven directly by the RNA polymerase II-dependent promoter. While, RNA-replicon DNA vaccine is a double-layered DNA/RNA construct. The RNA-replicon DNA vaccine plasmids include a full-length cytomegalovirus (CMV) promoter-driven expression cassette including replicase gene and heterologous protein. The first layer of this vector system, similar to conventional DNA vaccine construct, utilizes a RNA polymerase II-dependent promoter. But instead of driving the expression of the heterologous gene, it stimulates the transcription of the RNA transcript consist of RNA-replicon of replicase complex and heterologous gene, which is transported into cytoplasm. Translation of this RNA molecule produces the viral replicase complex, which catalyzes cytoplasmic self-amplification of recombinant RNA, and abundant subgenomic mRNA encoding the heterologous protein which subsequently yields high-level expression of the heterologous antigens. Self-replicating RNA is capable of replicating in a diverse range of cell types and allows the expression of heterologous antigen of interest at high levels.

Several groups have demonstrated the ability of RNA replicon based vaccines to induce high-level humoral and cell-mediated immunity against a variety of antigens, and the immunized animals developed more pronounced immune responses than those received a conventional DNA vaccine encoding the same antigen. These reports have also demonstrated that replicase-based nucleic acid vaccines require doses 100 to 1,000-times lower than conventional DNA plasmids to induce more prominent humoral and cell-mediated immune responses than those receiving a conventional under DNA vaccine encoding the same antigen. In general, studies showed that alphavirus

replicon vectors induced strong antibody and $CD8^+$ T-cell responses to encoded immunogens and in most cases protected immunized animals from appropriate virus challenge.

Enhancement of immunogenicity by activation of dsRNA-dependent antiviral defense

During the process of transgene amplification by replicase inside the cytoplasm, the negative-stranded anti-transgene RNA, full-length positive-stranded RNA and a shorter subgenomic RNA are produced. These RNA species form dsRNA molecules, as these are complimentary in sequences. The dsRNAs induce type I interferons and heat shock proteins, thus helping to initiate an immune response to a (perceived) viral infection. Consequently, RNase L and the PKR pathways are activated and induce apoptosis of transfected cells. Apoptosis has been shown to stimulate the immune system by uptake of apoptotic cells by dendritic cells, providing a potential mechanism for enhanced immunogenicity. Thus, the induction of apoptosis by replicase-based nucleic acid vaccines not only represents a welcome safety feature, but also seems to be critical for their function. Interfering with apoptosis by knocking out a pathway involved in inducing apoptosis after viral infection, such as the RNase L system, or reducing apoptosis by co-immunization with an anti-apoptotic gene significantly reduces vaccine efficacy. This indicates that apoptosis is desired feature of replicase-based DNA vaccines. As self-replicating DNA/RNA eventually causes lysis of transfected cells, these vectors therefore do not raise the concern associated with naked DNA vaccines of integration into the host genome.

Replicase-based nucleic acid vaccines do not over-produce antigen

Consequences of the transfection of cells with replicase-based DNA vaccine may account for this observation that transfected cells is not over producing antigen. The 2'-5'A synthetase/RNase L and the PKR pathways are activated by dsRNA and result in degradation of mRNA and blockade of protein synthesis, thereby limiting antigen production in cells transfected with replicase-based vaccines. Furthermore, replicase-based DNA vaccine can trigger apoptosis, which also helps in limiting over- production of antigen from the replicase-based vaccine.

Conventional and replicase-based DNA vaccines use different mechanisms

With the demonstration that the immunogenicity and efficacy of RNA-replicon-based DNA vaccines is improved significantly, not by providing more antigen for the adaptive immune response, but by delivering stronger 'adjuvant-type' signals to the innate immune system through activation of antiviral pathways. Together with immunostimulatory CpG motifs on bacterium-derived plasmids (recognized by Toll-like receptor 9, TLR9), the production of dsRNA by replicase-based nucleic acid vaccines (recognized by TLR3) may represent a potent stimulation of the innate immune system. The RNase L pathway is potentially the first of several innate (antiviral) pathways shown to be involved in the high immunogenicity of replicase-based DNA vaccines. Tapping into such innate pathways may be valuable to develop powerful vaccines while avoiding the side effects of highly immunogenic viruses or strong adjuvants.

Suggested Further Reading:

1. http://focosi.immunesig.org/RNAvaccines.html

2. http://www.genscript.com/gene_vaccine.html

3. http://www.modares.ac.ir/elearning/dna_rna_vaccies.htm

TLR LIGANDS – A CLASS OF NOVEL VACCINE ADJUVANTS

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Toll-like receptors are broadly distributed on the cells of the immune system such as macropages, dentritic cells (DC), neutrophils, B cells, as well as epithelical and endothelial cells.

TLRs in *Drosophila* Immunity

The first identified member of the Toll family, *Drosophila Toll*, was discovered as a maternal-effect gene that functions in a pathway that control dorsoventral axis formation of fruitfly embryos and later found to be associated with immunity.

TLRs in mammals

TLRs constitute a phylogenetically conserved family of PRRs that recognize and discriminate a diverse array of microbial antigens. At least 13 TLRs have been identified in mammals, and they detect a remarkably diverse array of bacterial, viral and fungal molecular pattens. TLRs are type I transmembrane receptors and are characterized by three structural features; a divergent ligand-binding extracellular domain with leucine-rich repeats, a short transmembrane domain and a highly homologous cytoplasmic toll/interleukin (IL-1) receptor domain (TIR), which is essential for initiation of downstream signaling cascades.

Functions of individual TLRs differ according to their expression pattern and signaling pathways. In addition to their ligand specificity, TLRs are differentially expressed by many distinct cell types, including monocytes/ mactophages/ microglia, dendritic cells, B cells, T cells, natural killer cells, and mast cells. In addition to the recognition of conserved microbial products which signal the presence of an infection and trigger required cellular immune response, TLRs also detect some endogenous ligands that might signal other danger conditions, such as degradation products of macromolecules, products of proteolytic cascades, intracellular componenets of ruptured cells and products of genes that are activated by inflammation. TLR3, -7, -8 and –9 recognize nucleic acids but the discrimination between nucleic acids of mammalian versus microbial origin by these TLRs needs further investigation. Interestingly, these TLRs are localized intracellularly and recognize nucleic acids in late endosomes-lysosomes. Under certain conditions, such as tissue injury or deficiency in clearing apoptotic cells, host-derived nucleic acids may become available to these TLRs and serve as endogenous ligands.

The inditification of TLR endogenous ligands challenges the traditional view that major functions of TLRs are considered to distinguish self-non-self but supports the 'danger-hypothesis' proposed my Matzinger, who suggested that the immune system has primarily evolved to recognize danger signals rather than non-self signals. Furthermore, the mechanisms of endogenous activation of mammalian TLRs may provide key insights for the understanding of multiple pathophysiological conditions following injury.

TLRs in mammalian immunity:

In mammalian species there are at least thirteen TLRs and each seems to have a distinct function in innate immune recognition. In the past few years, dozens of TLR ligands have been indentified.

Many more ligands are yet to be identififed. TLR ligands are quite diverse in structure and origin. However, several common themes are emerging based on the available information. First, most TLR ligands are conserved microbial products (PAMPs) that signal the presence of infection. Second, many and perhaps all, individual TLRs can recognize several, structurally unrelated ligands. Third, some TLRs required accessory proteins to recognize their ligands. Finally, although the actual mechanism of ligand recognition is still not known, available evidence indicates that mammalian TLRs recognize their ligands by direct binding and therefore function as PRRs.

TLRs and control of adaptive immunity

Specificity of the TLRs for products of microbial origin allows them to signal the presence of infection and to direct the adaptive immune responses against antigens of microbial origin.

TLR4

TLR4 functions as the signal-transducing receptor for LPS.This discovery was made by positional cloning of the *Lps* gene in the LPS-non-responsive C3H/HeJ mouse strain, and was confirmed in *Tlr4* knockout mice. C3H/HeJ mice are unresponsive to LPS due to a point mutation in the TIR domain of *Tlr4*, which abrogates downstream signaling.

In addition to LPS, TLR4 is involved in the recognition of several other ligands, including LTA, and a heat-sensitive cell-associated factor derived from *Mycobacterium tuberculosis*. TLR4 is also implicated in the recognition of the heat-shock protein HSP 60 which is conserved from bacteria to mammals. It is normally not available for recognition by cell-surface receptors, but presumably can be released from necrotic cells during tissue injury or lysis of virally infected cells. The physiological significance of HSP60 recognition by a TLR is not yet understood, but the inflammatory response induced by necrotic cells (which might be mediated by HSPs and other ligands released from dying cells) might have a role in tissue remodeling and wound healing.

TLR2:

TLR2 has been shown to be involved in the recognition of broad range of microbial products, including; peptidoglycan from Gram-positive bacteria. Bacterial lipoporotein, mycobacterial cell-wall lipoarbionomannan, glycosylphosphatidylinositol lipid from *Trypanosoma Cruzi* a phenol-soluble modulin produced by *Staphylococcus epidermidis*, and yeast cell walls. In addition, TLR2 functions as a receptor for atypical LPS produced by *Leptospira interrogans* and *Prophyromonas ginigivitis*, both of which are structurally different from Gram-negative LPS. This unusually broad range of ligands recognized by TLR2 is explained, in part, by cooperation between TLR2 and at least two other TLRs: TLR1 and TLR6.

It is interesting to note that both TLR1 and TLR6 are expressed constitutively on many cell types, whereas expression of TLR 2 is regulated and seems to be restricted to antigen-presenting cells and endothelial cells. The combinatorial recognition by TLR2 and regulation of its expression might provide an important mechanism to control cellular responsiveness to microbial products. The full repertoire of possible TLR heterodimers is not yet known, but TLR4 and TLR5, at least, are likely to function as homodimers.

TLR3:

TLR3 has interesting features that distinguish it from other mammalian TLRs. First, cloning of

human and mouse TLR3 showed that, unlike all other TLRs, TLR3 does not contain the conserved proline residue in the position equivalent to proline-712 of mouse TLR4. Substitution of this proline residue for histidine in the TLR4 gene in the C3H/Hej mouse strain results in unresponsiveness to LPS. Equivadlent substitutions in some other TLRs abolish their signaling activities. Therefore, the fact that TLR3 lacks the conserved proline at this crucial position indicated that the TLR3 signalling mechanism might differ from that of other TLRs. The second interesting feature of TLR3 is that it is expressed predominantly, albeit not exclusively, in dendritic cells.

Recent studies have shown that TLR3 functions as a cell-surface receptor for double-stranded RNA (dsRNA). DsRNA is a molecular pattern produced by most viruses at some point of their infectious cycle. Although the contribution of TLR3 to antiviral defence remains to be shown, the fact that dsRNA-an important viral PAMP – is recognized by a TLR, significantely broadens the range of pathogens that can be detected by the TLRs.

TLR5:

TLR5 is involved in recognition of flagellin-a conserved protein that forms bacterial flagella. An unusual aspect of this TLR ligand is that, unlike most other PAMPs, flagellin is a protein, and it does not undergo any post-translational modification that would distinguish it from host cellular proteins.

Interestingly, TLR5 is expressed on the basolateral side of the intestinal epithelium, where it can sense flaggellin from pathogenic bacteria, such as *Salmonella*. Polarized expression of TLR5 (and presumably other TLRs) on surface epithelia might provide an important mechanism of discrimination between commensal and pathogenic bacteria, as pathogenic, but not commensal microbes, can cross the epithelial barriers.

TLR9:

Perhaps the most enigmatic example of pattern recognition is the recognition of unmethylated CpG motifs in bacterial DNA by TLR9. Unmethylated DNA in a particular sequence context (the so-called 'CpG motif') has long been known for its potent immunostimulatory activity. A single nucleotide substitutuion or methylation of a cytosine residue within the CpG motif completely abrogates the immunostimulatory property of bacterial DNA, because bacteria lack cytosine methylation, and most CpG is methylated in the mammalian genome, CpG motifs might signal the presence of microbial infection. The essential role of TLR9 in CpG DNA recognition was shown using *Tlr9* knockout mice. Interestingly, signaling by CpG DNA requires its internalization into late endosomal or lysosomal compartments. The reason for this is not yet known, and it will be important to determine the subcellular localization of TLR9. It is not yet known whether any other TLR ligands need to be internalized in order to activate TLRs. Notably, TLR2 is expressed on the cell surface and is recruited to phagosomes on interaction with yeast cell walls (zymosan).

TLR signaling pathways

Activation of signal transduction pathways by TLRs leads to the induction of various genes that function in host defence, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules. Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthase and antimicrobial peptides, which can directly destroy microbial pathogens.

The recognition of a microbial molecule to its respective TLR, transmits a signal to the cell's nucleus initiating the expression of genes coding for the synthesis of regulatory molecules such as cytokines, chemokines, adhesion molecules etc., The cyctokines, in turn, bind the cytokine receptors on other defence cells. Cytokines such as IL-1, tumour necrosis factor (TNF)-2 and interferons, trigger innate immune defences and provide an immediate response against the invading micro-organism. However, excessive activation of these inflammatory cytokines leads to septic shock or sepsis, a leading cause of death in humans with bacterial infections. TLR also participate in adaptive immunity by triggering various secondary signals needed for humoral and cellmediated immunity.

Antigen-presenting mDC can be controlled at three points in the TLR cascade. Property of the adjuvant is the first to regulate TLR-mediated maturation of mDC. Secondly, modulation of TLR activity occurs by the addition of soluble forms of TLRs or TLR-blocking molecules. Finally, TLR signalling can be positively regulated by the adapters or negatively regulated by their inhibitors. Recent finding suggests that there are small synthetic molecules that block TLR-adapter interaction. Examples for the second and third control steps are as follows.

Alternatively spliced forms of TLR2, TLR4 and probably TLR3 appear to serve as dominant-negatives to block the relevant TLR activation in response to their ligands. Reports on this types of TLR regulation were published in human and mouse TLRs. Fish has a gene encoding a soluble TLR5, which acts as an amplifier of the flagellin-mediated membrane TLR5 signaling that induces acute phase cytokines as well as soluble TLR5. Although human has no gene for soluble TLR5, the amplification of flagellin signal by soluble TLR5 may be conserved. Fish soluble TLR5 can physiologically bind flagellin and augment the function of human membrane TLR5 in response to flagellin. The results may offer an adjuvant positively regulating flagellin response in human mDCs. Lipopolysaccharide-binding protein or soluble CD14 may upregulate LPS-mediated TLR4 activation. It is likely that yet to be indentified catch-up receptors for pattern molecules function as TLR modulators. Antagonists of TLRs either binding to the LRR or TIR domain of each TLR will be applicable for patients with inflammation or autoimmune status to block off TLR activity.

In the human kidney cell line HEK293 cells, overexpressed adapters enhance activation of corresponding pathways leading to the promoter activation even in the absence of adjuvant. Similarly, overexpressed dominant-negative forms of adapters effectively block the downstream signaling of adapters. In mDCs, adapter function can be positively regulated by the transfection of correspondingly associated adapters.

Vaccine adjuvant activity of TLR for NK activation

NK can be activated by type 1 IFNs and / or instructive cytokines, IL-12, IL-23 and IL-18. TLR3 engages in NK activation via TICAM-1-mediated signalling in mDCs. Thus, NK can be activated by mDCs that are pretreated with TLR3 agonist such as dsRNA. This means that mDC turns an activator for NK if appropriate TLR agonists are provided. In light of this, it appears that supplementing vaccine adjuvant with TLR3 to induce systemic activation of NK appears to be effective in humans with cancer or infectious diseses.

If one can supplement the adjuvant cocktail sufficiently to activate both CTL and NK, it would be possible to eliminate both MHC-postive and –negative target cells. Although such a scenario is largely extended from the mouse studies, the same mechanism of induction of antiviral immunity

appears to be the case in humans according to *in vitro* studies using human mDC. Viral infection often induces promotion of suppression of DC maturation. Supplement of TLR 2/4 agonists to viral dsRNA may relieve the maturation stages of DCs.

MyD88 is the adapter shared by receptors for IL-1B, IL-18 and most members of the TLR family. In mDCs, the transcription factor NF $-_{\kappa}$B is activated in the MyD88 pathway. MyD88 may support events other than those responsible for innate immune responses and the danger signal induced by tumors or virus-invaded tissue in effecter lymphocytes. In pDCs, the MyD88 pathway also activates IRF-7, which is followed by robust production of IFN. Type 1 IFN directly enhances the expression of IL-18 R components (AcPL), IL-IR-related protein (IL- 1 Rrp) and MyD88 in NK and T cells. This is reminiscent of the properties of the danger signal in the suppression of tumor cell progression or viral proliferation. It is reasonable to hypothesize that most danger signals suggest an enhanced effect of vaccine and activation of adapters. TICAM-1, another effective adapter, also induces activation of IFN-B and NF-$_{\kappa}$B in mDCs. It does not appear to function in pDCs fir IFN-x induction. TICAM-1 may be an important vaccine potentiator targeting mDCs. Further studies are needed to clarify the discerning properties of the two main adapters in terms of enhances for vaccine effect.

Adjuvants as ligands for TLRs

Freund's complete adjuvant (FCA) consisting of dead mycobacteria conjugated with mineral oil augments vaccine response, i.e. antibody (Ab) production, CTL induction and NK activation. Without the adjuvant, usually only a poor immune response is observed upon vaccination. Thus, the adjuvant has been an essential factory for provoking strong host immune responses. It has been elucidated that myeloid dendritic cells (mDCs), a representative cell population of antigen-presenting cells, and plasmacytoid dendritic cells (pDCs), formerly called type I interferon (IFN) – producing cells, are the targets for most of the adjuvants

Manipulation of TLR system for adjuvant vaccine therapy.

Effective therapeutic vaccines contain two primary constituents, antigen and adjuvant. Adjuvants consisting of microbial pattern molecules play a central role in vaccination. Successful vaccine requires efficient induction of antibody (Ab), type I interferons (IFN), cytokines, chemokines, cytotoxic T lymphocytes (CTL) and /or NK Cells. Toll-like receptors (TLRs) in myeloid dendritic cenlls (mDC) essentially act as adjuvant receptors and sustain the molecular basis of adjuvant activity. Current consensus is that TLRs and their adapters introduce signals to preferentially induce IFN-x/B, chemokines and proinflammatory cytokines, and mature mDC to augment antigen presentation. Although most of these data were obtained with mice, the results are presumed to be adaptable to humans. Whenever TLR paythway is activated in mDC, NK and/or CTL activation is promoted. For induction of antigen-specific CTL toward phagocytosed material, crosspriming must be induced in mDC, which is also sustained by TLR signaling in mDC. Since the TLR responses vary with different adjuvants, mDC functions are skewed depending on adjuvant-specific direction of mDC maturation. It appears that the directed maturation of mDC largely relies on selection of appropriate sets of TLRs and their adapter signaling pathways. Synthetic chimera molecules consisting of TLR agonists and target atingens are found to be effective in induction of CTL to eliminate target cells *in vivo*.

Table 1 Clinical development: vaccines and vaccine adjuvants

Indication	Compound	Target	Company	Status
Prophylactic				
Hepatitis B	Fendrix (HBV antigen and MPL adjuvant)	TLR4	Glaxo-Smithkine	Approved(EU)
Hepatitis B	Supervax (HBV antigen and synthetic MPL RC-529)	TLR4	Dynavax Technologies	Approved (Argentina)
Hepatitis B	Heplisav (HBV surface antigen and CpG-ODN 1018 ISS)	TLR9	Dynavax Technologies	Phase III
Human papillomavirus	Cervarix (HPV-16 and HPV-18 L1 Virus-like particles with aluminum hydroxide MPL adjuvant)	TLR4	Glaxo-Smithkline	Pre-approval
Anthrax	VaxImmune (CpG7909, a CpG B class ODN) with approved anthrax vaccine 9Biothrax)	TLR9	Coley pharmaceutical/DARPA	Phase I
Influenza	Fusion proteins of flagellin to hemaglutinin or M2e	TLR 5	Vaxinnate	Preclinical
Influenza	Influenza antigens and CpG-ODN	TLR 9	Dynavax Technologies	Preclinical
Human immuno-deficiency virus	HIV Gag protein coupled to imidazoquinoline compount 3M-012 (ref.53)	TLR7/8	NIH Vaccine Research Centre	Research
General Vaccine adjuvants	E6020 (syntheiic agonist)*.	TLR4	Eisai/Sanofi Pasteur	Preclinical
Therapeutic	Melan-A peptide in incomplete Freund adjuvant with CpG-ODN 7909	TLR9	Coley Pharmaceuticals/GSK	Phase I
Melanoma				
Non-small-cell-lung cancer	Stimuvax/BLP25 (synthetic cancer-associated MUC1 protein and MPL enclosed in liposomal vehicle)	TLR4	Biomera /Merck	Phase II
Melanoma	CYT004-MelQbG10 (Melan-A/Mart-1 protein coupled to immunodrug carrier QbG10)	TLR 9	Cytos Biotechnology	Phase II
Human immunodeficiency virus	Remune (inactivated HIV-1 virus) with HYB 2055 (CpG-ODN)	TLR 9	Idera Pharmaceuticals / Immune Response Corporation	Phase I/II

Clinical development of TLR agonists

Vaccine adjuvants are perhaps the most extensively explored applications for TLR agonists. The rational design of specific TLR agonists with reduced toxicity but increased potency, as compared to adjuvant candidates from only a decade ago, offers the opportunity to meet the stringent safety criteria required for prophylactic vaccines. APC, primarily dendritic cells, are the principal cellular targets, as they constitute the link between innate and subsequent adaptive immune responses. As present, two improved adult HBV vaccines that use TLR4 agonists as the adjuvant have been approved.

TLR agonists also feature prominently in efforts to develop therapeutic vaccines against cancer and chronic viral diseases. The potential of certain TLR ligands to facilitate these challenging vaccines is twofold: (i) enhancement of CD8+ T-Cell responses to protein antigens, which requires cross-presentation of peptides generated from exogenous antigens and (ii) overcoming tolerance to self-antigens, probably necessary for generating responses to tumor-associated antigens that have few differences from normal self-antigens.

Encouraging results have been reported in humans with an anti-tumor peptide vaccine using a CpG-ODN as adjuvant confirming findings in many different mouse tumor models. Preclinical studies suggest that TLR3 TLR4, TLR7 and TLR 7/8 agonists also have potential to enhance therapeutic vaccination for cancer and chronic viral infections, including HIV and HBV.

Further enhancement can be achieved by optimizing co-delivery of the virus or tumor antigen with a TLR agonist. This was initially demonstrated by convalently linking CpG-ODN to ovalbumin in a mouse model, leading to stronger CD8+ T cell priming compared to that with a CpG-ODN and antigen mix. Similarly, conjugation of HIV Gag protein to a TLR7/8 agonist substantially enchances CD8+ T cells in nonhuman primates. Linkage of a TLR agonist to antigen can increase antigen uptake by DC, reducing the needed antigen does. Simultaneous delivery of adjuvant the protein may also facilitate antigen processing and MHC class I and II antigen presentation. Moreover, adjuvant activity of most TLR agonists can be enhanced by formulation in lipid emulsions, microparticles or virus-like particles containing antigens.

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COWPATHY FOR IMMUNOMODULATION OF VACCINE RESPONSE

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Cowpathy (Panchgavya chikitsa) is an age-old system of medicine described in ancient Indian literature 'Ayurveda', means the therapy of human ailments through cow products. In Ayurvedic system, the medicines are prepared either from plants or from animals besides the use of certain metals. The Ayurvedic medicines of animal origin are mainly prepared from indigenous cow products such as urine, dung, milk, curd and ghee. Medicinal use of cow urine has been in practice since time immemorial in India. The Panchgavya principle of Ayurveda consists of cow urine besides other products like milk, ghee, buttermilk (matha), dung as its main ingredients. The preliminary studies on immunomodulation with cow urine generated interest among the scientists. The cow urine distillate (Kamdhenu Ark) was found to increase immunity in mice. It also increases the phagocytic activity of macrophages and secretion of interleukin 1 and 2. Recently, the cow urine has also been granted US patent for its synergistic properties with antibiotics and as bioenhancer. It provided the base for further research and detailed studies on immunomodulatory properties of indigenous cow urine and its comparison with the urine of cross bred and exotic cows, buffaloes and goat using modern biotechnological tools. As per an estimate by WHO, by the year 2020, the antibiotics will no more be wonder drugs. It is an established fact that most of the antibiotic drugs have lost their capacity by way of increased resistance in bacteria. Then in such situation, cowpathy will play a major role in prevention and control of infections in man and animal.

The Indian cow

The indigenous cattle, scientifically called as *Bos indicus* or as Zebu cattle, mainly inhabitat the Indian subcontinent. It is thought to be world's oldest domesticated cattle. Historically also it is now proved by the fact that humped cattle remains were found in Mohanjodaro site of Indus Valley indicating their presence in India even before the arrival of Aryans. Presently, cow rearing is an important source of income and an enterprise, which enables poor and landless farmers to earn income using common property resources and land. The cattle are fed on crop residues and farm produce by products that would otherwise be wasted, and as such there is no food competition with human beings.

Importance of cow urine

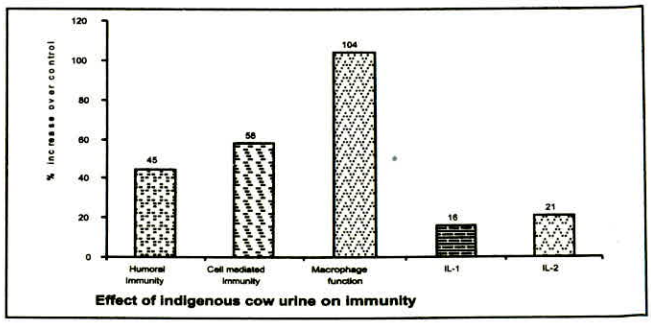
Cow urine has many beneficial properties particularly in the area of agriculture and therapeutics. It has also been observed in scientific research that the urine of Indian cows is highly effective as compare to the urine of other species. It is a good biopesticide and also effective against many diseases including cancer. It is a very potent immunoenhancer. In 'Sushruta Samhita' and Ashtanga Sangraha' cow urine has been described as the most effective substance/secretion of animal origin with innumerable therapeutic values. Urine of cow contains all the beneficial elements so it is natural and universal medicine that fulfills the deficiency of the elements in the body. Cow urine contains 24 types of salts and the medicines made from cow urine are capable of curing even the most incurable diseases. Cow urine contains 95% water, 2.5% urea, and 2.5% minerals, salts, hormones and enzymes. It contains iron, calcium, phosphorus, salts, carbonic acid, potash,

nitrogen, ammonia, manganese, sulphor, phosphate, potassium, urea, uric acid, amino acids, enzymes, cytokines, lactose etc. Cytokines and amino acids may play a role in immunoenhancement.

Most of the medicines are made by distilling urine and collecting vapors known as ark or distillate. A number of ailments could be treated and it is being used even for the most threatening diseases like cancer, AIDS, diabetes and skin problems. Cow urine is also antibacterial, antifungal, antiviral, antineoplastic, anticonvulsant, antispasmodic and still non-toxic. It is also beneficial in conditions like flu, sinus, allergy, cold, ear infection, rheumatoid arthritis, aging, bacterial/viral infection, snake bites, chemical intoxication, chicken pox, enteritis, constipation, edema, baldness, hepatitis, leprosy, hypertension, burns, tuberculosis, asthma, tetanus, Parkinson's disease, obesity, gastric ulcers, depression, heart diseases, fever, eczema fatigue etc. It is also used as diuretic, laxative and for treatment of chronic malaria, headache and fever. It is a proven universal cure for the blood disorders, leucorrhoea and even leprosy. The urine of cow is bitter, pungent, spicy, warm, and full of all the five elixirs. In India drinking of cow urine is in practice since thousands of years and now it has also been demonstrated experimentally that it is one of the best natural medicines in the universe.

Cow urine as an immunomodulator

Outside the India, there is no research work reported in literature as far as the cowpathy or medicinal properties of cow urine are concerned. However, there is a US patent issued to inventors Khanuja *et al.* vide on. 6410059 dated 25.6.2002 on a pharmaceutical composition comprising of an antibiotic and cow urine distillate in an amount effective to enhance antimicrobial effect of antibiotics. In India, the ancient literatures including Ayurveda have description on Panchgavya therapy that includes cow urine, milk, curd, ghee and dung. The Panchgavya therapy though an age-old system of medicine is not given due importance in modern science. However, there are scanty reports of therapeutic use of cow urine or other Panchgavya materials to cure human and animal ailments. Immunomodulatory properties of cow urine distillate in mice recorded an increase in humoral and cellular immunity of 45% and 59%, respectively. The parameters used to assess immunity were B-lymphocyte blastogenesis, T-lymphocyte blastogenesis, serum IgG and IgM levels. The cow urine also stimulated the production of interleukin 1 and 2 by 16% and 21%, respectively, from peripheral blood leucocytes of mice. The phagocytic activity of macrophages was enhanced by 104% in mice treated with cow urine in comparison to controls. Panchgavya is considered as a wonder formulation, which requires scientific validation using modern biotechnological tools in order to increase its acceptance in the society. The cow urine distillate was also found to be a good immunostimulant in comparison to Vasant Kusmakar in mice. The cow urine was given orally to a female patient having intestinal cancer for a period of 4 months, and it provided relief to the patient by reducing the motions, hemorrhage and increased activity of the patient. Distilled cow urine has been found to increase the humoral immunity in rats. Lymphocytes



Effect of indigenous cow urine on immunity

proliferation in response to mitogen in the developing chick embryo increased with the use of cow urine. This means that immune system developed at an early stage and embryonic mortality can be decreased with the use of cow urine. Urine of red hill cow, found in Uttaranchal state and characterized as *Badri cow* has been found to be most potent immunostimulator as compared with the urine from other animals including indigenous cow (Sahiwal), goats, buffalo (Murah), cross bred cow (Sahiwal x Jerssey) and exotic cows (HF). It was observed that urine of crossbred, exotic cow and buffalo has no immunomodulatory effect. Cow urine given to the poultry birds in water as an alternative to antibiotics demonstrated excellent immunomodulatory properties in addition to the increase in the egg production and egg quality of the layer birds. In another important study effect of cow urine on the lymphocytes damaged by pesticides was observed. It was found that cow urine decreases the apoptosis caused by the heavy metals in avian lymphocytes. Thus, corroborating to some extent with the findings that cow urine help in repair of broken DNA. The antioxidant properties of cow urine distillate include protection or DNA and its repairs. The cow urine distillate protected the chromosomal aberrations caused by mitomycin-C in human leucocyte culture. Similarly, cow urine was found to be a very good antioxidant. Cow urine has a high antioxidant status as indicated by its ability to destroy the free radicals. If we look into the fact of apoptosis, it starts with fragmentation of nucleic acid (DNA) into oligonucleotides of 200-300 bp. Several studies carried out by the authors and others at Pantnagar, NEERI Nagpur and CIMAP Lucknow are suggestive of its properties to repair damaged DNA and thereby protecting cells from suicidal activity enhanced due to pesticides/any other harmful chemical residues.

Harmful effects of pesticides and their prevention through cow urine

In the thirst of modernization and industrialization, man has contributed pollution in the environment leading to disturbance in life cycles and ecology of plants, animals and microbes. Increased demand for food and fibre has lead to the chemicalization of agriculture and we have reached to such a stage that modern agriculture is dependent on high yielding varieties, which can only be grown under the influence of fertilizers and pesticides. Chemical pesticides, owing to their universality and diverse effects, are widely used in agriculture to protect crops from pest menace. Repeated application of pesticides and fertilizers resulted in contamination of various crops with their residues, metabolites and/or heavy metals, which are having easy access into the food chain. The main pesticides, which are used in agriculture, animal husbandry or public health operations, are classified into 4 groups along with their percent share in market (i) Insecticide 77.0%, (ii) Herbicide 13.0%, (iii) Fungicide 8.6%, (iv) Rodenticide 1.0%, (v) Others 0.4%.

Majority of these pesticides are beneficial when used for specific purposes, handled properly and applied as per the recommendations of the manufacturer. However, over the years there has been a mounting fear and concern that indiscriminate and improportionate use of pesticides may lead to harmful effects in man and animals who are exposed to the low level of pesticide residues in air, water and food chain. In an ideal pesticide application, the chemical should fall exactly on the target and be degraded completely to harmless compounds. However, this situation is never attained practically and only some part of the pesticide hits the target pests while remaining drifts into environment.

The presence of pesticide residues has been detected in various items including food and feeds. Similarly, heavy metals such as lead, mercury and cadmium, which are common contaminants of pesticides and/or fertilizers, may get entry into the food chain. The levels of

pesticides and heavy metals in food items are found to be at much higher levels than expected, because of heavy contamination of the environment.

In past, most of the research work had been directed towards the clinical toxicity of the pesticides/heavy metals but little attention has been paid to study the indirect toxicity using chronic low doses. However, there have been many reports of breakdown of immunity of animals and/or man due to pesticides and inspite of proper vaccination, diseases do occur in vaccinated population. Though various reasons have been suggested as cause of vaccinal failure but there seems to be an important factor associated with pesticide residues in food chain that leads to immunodeficiency in animals.

Experiments were planned to study the effect of cow urine on B- and T-lymphocytes treated with pesticides and the regents are quite encouraging (Table 1 & 2). Cow urine protected he cells upto 55% from the deleterious effects of pesticides.

Table 1. Effect of cow urine on B-lymphocytes treated with pesticides

Pesticides	B-lymphocytes (%)	Treated with cow urine (%)	Protection due to cow urine (%)
Cypermethrin	56	16	40
Allethrin	92	60	32
Captan	87	45	42
Dimethoate	73	18	55
Mthyl parathion	87	54	33
Forate	81	58	23
ncozeb	60	29	31
Prpoxur	76	65	11
Thriam	61	39	22
Zineb	83	68	15

Table 2. Effect of cow urine on T-lymphocytes treated with cow urine

Pesticides	T-lymphocytes (%)	Treated with cow urine (%)	Protection due to cow urine (%)
Cypermethrin	56	47	9
Allethrin	92	57	35
Captan	87	51	36
Dimethoate	87	20	67
Methyl parathion	68	55	13
Forate	82	67	15
Mancozeb	55	36	14
Propoxur	71	68	3
Thriam	67	35	32
Zineb	87	71	16

Comparison of urine of animals

The urine of indigenous cow is also compared with the urine of other animals such as crossbred cows, buffaloes, goats, exotic cows and hill cows. It has been observed that the urine

of indigenous and hill cows is quite effective as far as the immunomodulation is concerned the goat urine is also effective but upto the 50% of the cow urine. This finding is further supported by the presence of "Rasayan" in the urine of indigenous cows (Table 3).

Table 3. Analysis of urine of animals through chemical fingerprinting (HPLC)

	Characteristics	Indigenous cow	Hill cow	Goat	Exotic cow	Crossbred cow	Buffalo
1.	Tridos har	√	√	√	√	X	√
2.	Madhur ras	√	√	√	√	X	X
3.	Madhur vipak	√	√	√	X	X	√
4.	Katu ras	√	√	√	√	X	√
5.	Tikta ras	√	√	√	X	√	√
6.	Kashay ras	√	√	√	√	√	√
7.	Raktas shodhak	√	√	√	√	√	√
8.	Deepan	√	√	√	√	√	√
9.	Pachan	√	√	√	X	√	√
10.	Rasayan	√	√	√	X	X	X
11.	Amhar	X	√	√	√	√	√
12.	Vat viridhi	√	√	√	√	X	X
13.	Hepatoprotective	√	√	√	√	X	√
14.	Stress reliever	√	√	√	√	X	√
15.	Effect on blood calcium level	√	X	X	√	X	X

Future prospects

Cow urine has immense potential of being used as an immunomodulator particularly along with antibiotics and/or vaccines in order to enhance their activity. However, its palatability in crude form as it is being prepared and marketed by several organizations, is not much accepted in the society. Therefore, the efforts are being made to prepare the dry form of cow urine without loosing its activity but changing the delivery system. Another area is to study the urine of Indigenous cows of different breeds in various geoclimatic zones at different nutrition levels. In future, it can be given as a 'biovaccine' to protect man and animals from various diseases.

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RECENT DEVELOPMENTS IN THE ANIMAL VIRUS VACCINES

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INTRODUCTION

The terms vaccination and vaccine derive from the work of Edward Jenner who, over 200 years ago, showed that inoculating people with material from skin lesions caused by cowpox (L.vaccinus, of cows) protected them from the highly contagious and frequently fatal disease smallpox. Since Jenner's time the term has been retained for any preparation of dead or weakened pathogens, or their products, that when introduced into the body, stimulates the production of protective antibodies or T cells without causing the disease. In molecular terms, the goal is to introduce harmless antigen (s) with epitopes that are also found on the pathogen. Vaccination is also called active immunization because the immune system is stimulated to develop its own immunity against the pathogen. Passive immunity, in contrast, results from the injection of antibodies formed by another animal (e.g. horse, human etc.) which provide immediate, but temporary, protection for the recipient. Vaccine is a preparation containing a pathogen in either attenuated or inactivated state or an antigen from pathogen. A vaccine is collection of immunological determinants which are presented to the immune system as killed or live antigen which provoke protective immune responses. It is a non-pathogenic antigen that mimics a particular pathogen in order to elicit an immune response as if that actual pathogen were in the body. The overall goal of a vaccine is to establish immunity against that particular pathogen.

SOME OF THE TRIUMPHS OF VACCINATION

The greatest triumph is the eradication of smallpox from the planet, with no naturally-occurring cases having been found since 1977. "Naturally-occurring" because one case (fatal) occurred later following the accidental release of the virus in a laboratory. As far as the public knows, smallpox virus now exists only in laboratories in the U.S. and Russia. There is currently a vigorous debate as to weather these should be destroyed. If smallpox ever should get back out into the environment, the results could be devastating because smallpox vaccination is no longer given and so the population fully susceptible to the disease grows year by year.

Viral Vaccines

The viral vaccines are broadly categorized in to live attenuated virus vaccines, inactivated or killed vaccines and vaccines produced by modern Biotechnology. Biotechnological Approaches to develop new generation vaccine includes

- Recombinant Subunit Vaccines
- Vectored Vaccines
- Synthetic Peptide Vaccines
- Genetically altered Live Vaccines

- DNA vaccines (Naked nucleic acid Vaccine)
- Anti-idiotype Vaccine
- Plant and Plant Viruses based Vaccines

Live Attenuated Vaccines

In these vaccines, the virus can still infect but has been so weakened that it is no longer dangerous.

Advantages

- Single dose may be effective
- Can be given by any route.
- Produces long lived immunity
- Inexpensive

Disadvantages

- Possible reversion to virulence
- Possible spread to in contact animals and fetus
- Possible contaminating viruses or Mycoplasma

Inactivated or Killed vaccine

Like killed bacterial vaccines, these vaccines contain whole virus particles that have been treated so that they cannot infect the host's cells but still retain some unaltered epitopes.

Advantages

- Stability
- No danger of spread
- No problem with viral interference

" Disadvantages

- Multiple doses often required to protect
- No local immunity or interferon produced
- High concentration of antigen causes them to be expensive
- Immunity often short lived
- Properly non-inactivated virus can cause disease

Vaccines developed by recombinant DNA technology

Recombinant DNA technology for modification of bio-molecules was introduced in 1970 by Stanley Cohen and Herber Boyer. Recombinant DNA technology refers to the isolation of individual genes or group of genes and their insertion in to the genome of other organisms. DNA cloning and Genetic engineering involve the cleavage of DNA and assembly of DNA segments in new combinations -Recombinant DNA. Recombinant DNA technology has made it possible to retain the advan-

tages while overcoming the safety hazards and logistic.

Steps in Recombinant DNA technology

- Cutting DNA at precise locations. Sequence-specific endonuclease (Restriction endonuclease) provide the molecular scissors.
- Selecting a small molecule of DNA capable of self replication. These DNAs are called Cloning Vectors.
- Joining two DNA fragments covalently by Enzyme DNA ligase links the cloning vector and DNA to be cloned.
- DNA molecules comprising covalently linked segments from two or more sources are called recombinant DNAs.
- Moving recombinant DNA from the test tube to a host cell that will provide the enzymatic machinery for DNA replication.

Cloning vector

A cloning vector is a DNA molecule that has the ability to replicate in an appropriate host cells. There are numbers of cloning vectors have been developed by several workers and available commercially. The most commonly used cloning vectors used for recombinant DNA technology are as follows

- Bacterial Plasmid vector
- Cosmid vector
- Bacteriophages
- Vaccinia virus vector (using bacteria)
- Retro virus vector
- Bacculo virus vector etc

Host cells

It will provide the enzymatic machinery for DNA replication.

- Bacteria
- Tissue culture
- Insects (Silkworm)
- Animals

Recombinant Subunit Vaccines

The principle of recombinant DNA vaccines involves identifying the genome of the protective antigens, isolating it, inserting it into a vector and introducing it into a suitable host. The transplanted gene will be expressed and protein of interest will be synthesized. The protein can be separated purified and used as vaccine.

Advantages of Recombinant subunit vaccines

- Element of safety.
- Lack infectivity.
- Eliminating pyrogenic, allergic,immunosuppressive and other undesirable reactogenicity.
- A great deal of stability.
- The VLPs lack the genetic material hence can not pass on to the foetus.
- One can visualize the potential of the technology to produce vaccine chimeras representing different serotypes.

Recombinant Subunit Vaccines against Viral Diseases

Numbers of recombinant subunit vaccines against viral diseases have been developed for human and veterinary use with its own limitation and success. The first recombinant subunit vaccine approved for human use was the Hepatitis B vaccine. First successful recombinant vaccine of veterinary use was developed for the major immunogen VP1 of FMD virus. VP1 of FMDV is an ideal target antigen for the development of a subunit vaccine against FMD. The neutralizing immune response appears to be directed primarily against VP1. VPI gene carries type and strain specificities, receptor binding site, T cell epitopes and immunogenic sites Recombinant subunit vaccine against rabies has also been successfully used to protect foxes against rabies. Rhabdoviridae possess an immunogenic surface glycoprotein G (haemagglutnin). Rabies virus subunits or the isolated glycoprotein G alone elicited neutralizing antibody and induced immunity in animals. Recombinant Vaccinia virus expressing the glycoprotein G of rabies produce neutralizing antibody against rabies virus.

Purified VP2 protein of bluetongue virus has also been found to be effective to induce protective immune response against BTV. The VP2 obtained through recombinant baculovirus also elicited immune response that protected the immunised sheep against homologous virulent virus challenge. VLPs of rotavirus have also been produced by coinfecting insect cells with bacculovirus recombinants expressing rotavirus structural proteins VP1, VP4, VP6 and VP7 produces protective immunity against rota virus. Recombinant subunit vaccine has also been demonstrated for Parvovirus infection, Herpes Simplex 1 and, IBR, Bovine Leukemia, Rinderpest and HIV infection

Vectored Vaccines

Expression of heterogeneous genes into an avirulent viral / bacterial organism which are harmless to animals and induce immune response against foreign product can be used to protect the animals against a particular pathogen. Expression of more than one foreign gene encoding immunogenic proteins paves the way for development of multivalent recombinant DNA vaccine against human and animal viral infections. The most extensively used viral vectors include Vaccinia virus Vector, Pox viruses such as Capri pox, Fowl pox and Cannary pox viruses, Adenovirus, Herpes virus, Papova virus etc. Vectored vaccines against viral diseases are generally based on infectious, semi-infectious viral /bacterial vectors into which the genes of interest have been cloned. A number of relevant genes of other viruses have been also expressed in a variety of vectors and their vaccine potential has been studied extensively. Vaccinia virus has been used as a vector for development of vaccines against Rabies and Rinderpest.

Vaccinia virus Vectored viral vaccine

Name of the Disease	Etiological agent/ gene product	References
Herpes gcnital	Herpes simplex virus 1, 2/gB, gD, gG	Townsend K *et al.* , 1997
		Sullivan V *et al.* , 1987
Hepatitis B	Hepatitis Virus/S, MS, LS, Core	Cremer *et al.* , 1985
		Cheng *et al.* , 1987
AIDS	Human immunodeficiency virus, gp 160, gp 120, gp4I, gag,3'ORF	Clarke *et al.* , 1988
Influenza	Influenza virus, PBI, PB2, PA, HA, NA, NSI, NS2, NP, MI, M2	King AMQ *et al.* , 1987
Rota - gastroenteritis	Rotavirus SA-II /VP7	Murphy *et al.* , 1987
Rabies	Rabies virus/G, N	Smith GL *et al.* , 1987
		Esposito JJ *et al.* , 1987
Dengue fever	Dengue virus/C, NS I, prM/M, E	Esposito .1.1.*et al.* , 1988
Foot-and-Mouth	Foot-and-Mouth disease virus VPI	Cheng KC *et al.* , 1987
Rift Valley fever	Rift Valley fever virus G 1 ,G2	Zhao B *et al.* ,1887
Vesicular stomatitis	Vesicular stomatitis virusG,N 76	Deubel V *et al.* ,1992
Swine influenza	Swine influenza virusH 1	Collett M S *et al.* ,1987
Bluetongue	Bluetongue Virus VP2	Mackett M *et al.* ,1985
Rinderpest	Rinderpest Virus F protein	Gilbert J.H. *et al.* ,1987
Peste des petitis	Peste des petites virus F & H	Barrett T *et al.*. ,1989
Canine distemper	Canine distemper virus	Yilma T *et al.* ,1988

Synthetic peptide vaccines

Chemical synthesis of specific viral antigens may provide another important method of vaccine development. Small synthetic peptide having sequences of immunogenic epitopes on a protein will react with antibody to the intact native antigen and in some cases neutralize the biological activity. The understanding the only a few epitopes on structural proteins of viruses play an important role in inducing immune responses led to the development of synthetic peptide Vaccine. Computerized analysis of the amino acid sequence of the hepatitis B virus surface antigen was used to predict the location of an antigenic epitope in a limited portion of the molecule comprising residues 138149. This sequence was synthesized and its ability to bind the hepatitis B virus specific antibodies was tested. Result indicated that the synthetic peptide has ability to bind with the specific antibodies. Extensive research has been done to develop synthetic peptide vaccines against a number of viral diseases including FMDV and Influenza.

Advantages of synthetic Peptide vaccines

- It would have an indefinite shelf life, even at the temperatures in tropical countries.
- It would have precise composition.

- There would be no possibility of the adventitious presence of live virus.
- Special containment facilities necessary for handling large amounts of highly infectious virus would not be required.

Genetically altered live vaccines (Deletion mutant vaccines)

This is an attempt to attenuate a virus by deleting or altering the virulent gene by the use of rDNA technology to reduce its virulence or makes it unable to replicate completely and thus make live vaccines with precisely defined modifications. The removal of pathogenic genes from infectious agents without destroying their ability to propagate and express immuno dominant epitopes makes it suitable vaccine candidate for immunization of animals against viral diseases. Some exakples of gene deleted vaccines:

- A strain of pseudorabies virus has been attenuated by knocking out thymidine kinase gene using rDNA techniques and licensed.
- A live Simian Immunodeficiency Virus (SIV) vaccine, attenuated by deleting the nef gene, exhibited the most impressive protection against SIV in macaques challenged with highly virulent SIV after two years of immunization.
- Similar approach has been used to develop vaccine against bovine rhinotracheitis (IBR), Influenza and Rota virus infection.

Naked nucleic acid vaccines

Naked nucleic acid vaccines are the third generation vaccines where the naked plasmid DNA containing a marker gene could express itself when into the suitable animal host. Effective means of generating an antigen specific immune response by simply inoculating Plasmid DNA encoding viral genes. Delivery of DNA genes requires expression of immunogenic proteins in tissue accessible to the immune system such as muscle, skin, and mucus membrane. These vaccines are usually delivered by injection or by a device known as gene gun. The nucleic acid vaccine consists of the foreign gene of interest cloned into a bacterial Plasmid.

Advantage of DNA vaccines

- They induce both humoral and cellular immune responses, being CMI as predominant one.
- This class of vaccines follow the MHC class I pathway.
- They are easy to administer.
- There is no fear of neutralization by maternal antibodies in neonates.
- Their cost of production is less, and there is no requirement of maintenance of cold chain.

Anti-idiotypic vaccines

An antibody against a particular antigen when inoculated into phylogenetically unrelated animals elicits antibodies which behave like an antigen. Anti-idiotype antibodies may have in the antibody combining sites structure that mimic the structures of the antigen against which the original antibody was directed. Anti-idiotype antibodies can carry the image of the foreign antigen and act like it. Injection of the anti-idiotype antibodies in animals can produce antibodies that react to the original antigen. Thus anti-idiotype antibodies can be used as vaccines. Animals modulate the

response to a foreign antigen by the production 'of a cascade' of idiotype, anti-idiotype, anti-anti-idiotype and higher anti-idiotype antibodies. Anti-idiotype, antibodies can now be produced in large quantities in hybridomas or by cloning and expression of DNA transcribed from anti-idiotype antibody specific mRNA. Anti-idiotype antibodies have been produced against Parvovirus Newcastle disease virus and FMD Virus.

Candidate Viral DNA vaccines

Name of the disease	Etiological agent	References
Herpes genital	Herpes simplex virus 1	Chow *et al.*, 1997
Lymphocytic choriomeningitis	Lymphocytic choriomeningitis virus	Manickan *et al.*, 1995
		Martin *et al.*, 1995
Hepatitis B	Hepatitis virus	Yokoyama *et al.*, 1995
AIDS	Human immunodeficiency Virus	Zarozinsky *et al.*, 1995
Influenza	Influenza Virus	Xiang *et al.*, 1994
Rota-gastroenteritis	Rota virus	Herrman *et al.*, 1996
Rabies	Rabies Virus	Simmonds *et al.*, 1997
IBR	Bovine herpes virus	Osterrieder *et al.*, 1995
Equine rhinotracheitis	Equine herpes virus	Ulmer *et al.*, 1993
Name of the disease	Etiological agent	References
Foot and Mouth disease (FMD)	FMD virus	Zhang *et al*.,2003
Infectious bursal disease (IBD)	IBD virus	Wang-xiao quan *et al.*, 2003
Avian Influenza	Avian Influenza virus	Cherbonnel *et al.*, 2003
Dengue Fever	Flavivirus	Konishi *et al.*, 2003
Infectious laryngotracheitis	ILT Virus	Meng-song shu *et al*., 2002
Pseudorabies	Pseudorabies virus	Hong wen Zhon *et al.*, 2002

Edible Vaccines (Plant and Plant viruses based vaccines)

These are the vaccines produced from transgenic plants in which an active antigen of the target pathogen is expressed and accumulated which can give protective immunity against the particular pathogen when fed to the animals. Edible vaccines are prepared by introducing bacterial or viral genes coding for antigens into desired plants with the help of electric impulses or the particle bombardment or the vaccume infiltration or by direct DNA transfer.

Plants/Vegetables/ Fruits for Edible vaccines

- Banana May, G.D. *et al.* (1995)
- Tomato Sandhu, J.S. *et al.* (2000)
- Potato Tacket, C.O. *et al.* (2000)
- Peanuts Daniell, H. *et al.* (2001)
- Rice Daniell, H. *et al.* (2001)

- Wheat Daniell, H. *et al.* (2002)
- Corn Kusnadi, A.R. *et al.* (1997)
- Soyabean Kusnadi, A.R. *et al.* (1997)
- Carrots Daniell, H. *et al.* (2002)

Advantages of plant system in the development of oral vaccines

- Edible plants are very effective as a delivery vehicle for inducing oral immunization
- Adjuvant for immune response is not necessary
- Excellent safety and economic feasibility of oral administration compared to injection
- Easy for separation and purification of vaccines from plant materials
- Effective prevention of pathogenic contamination from animal cells
- Convenience and safety in storing and transporting vaccines
- Effective maintenance of vaccine activity by controlling the temperature in plant cultivation
- Easy for mass production system by breeding compared to an animal system
- Possible production of vaccines with low costs
- Edible means of administration
- Reduced need for medical personnel and sterile injection conditions
- Economical to mass produce and transport
- Reduced dependence on foreign supply
- Storage near the site of use
- Heat stable, eliminating the need for refrigeration
- Antigen protection through bioencapsulation
- Subunit vaccine (not attenuated pathogens) means improved safety
- Seroconversion in the presence of maternal antibodies
- Generation of systemic and mucosal immunity
- Enhanced compliance (especially in children)
- Delivery of multiple antigens

Edible Vaccines against Viral Diseases

- A highly successful example is the hepatitis B vaccine, which uses HBsAg expressed in transgenic yeast.
- HBsAg is the main envelope protein of hepatitis B virus (HBV), and is an integral membrane protein of the endoplasmic reticulum (ER).
- When expressed in transgenic tobacco, HBsAg makes VLPs with an average size of 22 nm.
- The plant-derived HBsAg was structurally similar to VLPs produced in transgenic yeast which is used as hepatitis B vaccine.
- A corn based an oral transmissible gastroenteritis virus vaccine boosts immunity in Swine

- Herpes simplex virus in transgenic soybeans.
- Capsid protein of FMD virus in transgenic Arabidopsis thaliana.
- Norwalk virus Capsid protein in transgenic tobacco and potato was successfully demonstrated.
- The other organisms mostly targeted by edible vaccine were Rota virus.
- Plant viruses have also been identified as potential vector for expression of foreign antigens and development of novel vaccines.
- Cowpea mosaic virus (CPMV) is one of them because of several important features of this virus.
- FMDV and HIV epitopes were expressed in CVPs (Chimeric virus particles) of CPMV which produced specific antibody response.
- Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1.
- A subunit vaccine using the TGEV envelope spike (S) protein was produced in transgenic corn and fed to piglets, resulting in 50% of virus-challenged animals being free of diarrhea.

Oral vaccine using a plant system

Most vaccine-developing technologies of remixing genes use revealed systems of animal cells and certain conditions of the cultivation of animal cells. Also, a disease virus is various and there are many variants so we cannot develop vaccine without paying a great deal of expenses to research and develop such vaccine. But the workers had already kept producing technology of vaccine including transgenic plant, transgenic vector production, and control of genetic revelation, separation and refinement of remixing protein analysis of transgenic plants, producing an antibody and separating proteins, and examination technology of the cause of an animal immunity and others. We can produce a great deal of safe vaccine from vegetable cells at a low price. The technology of producing vaccine using vegetable systems has many good points and has attracted public attention as the technology of developing effective vaccine a (covering the introduction of producing oral vaccine from plants).

The fruit delivery system: a new way to vaccinate

Vaccines produced in raw foods such as bananas may be a cost-effective alternative for controlling important diseases in developing countries. The proteins targeted for use in subunit vaccines are antigens, which may be thought of as the molecular signature of the pathogen. In viruses, antigens are usually proteins which appear on the surface of the virus and are well recognised by the immune system. If humans are exposed to an antigen, they recognise it as being a foreign molecule and develop an immune response against it, so, if exposed to the real bacteria or virus, their system can mount an effective and protective defence.

Conclusions

- The development of vaccines against viral diseases is one of the great achievements of human endeavors and science of Vaccinology has gone under revolutionary changes.

- Efforts have been made for developing more efficacious, safer, thermostable and economically affordable vaccines using molecular approaches.
- Recombinant vaccines have already been commercialized and some are under different stages of development.
- The promising area with future potential is expression of genes encoding immunogenic protein in plants and plant viruses.
- Naked nucleic acid vaccines also hold very promising future.
- Biotechnologically designed vaccines would allow the production of bivalent and even multivalent vaccines that are easier to produce and administer and have increased safety, efficacy, and potency.
- Biotech vaccines are generally expected to be more stable and heat resistant, so that low temperature would not be required.
- On going researches on Edible vaccines will definitely come up with a safest way to immunize children and animals. Get ready to eat a VACCINE BANANA.

SESSION - IV

Design & Development of Molecular and/or Immunodiagnostics

Chairman : **Dr. Lal Krishna**

Co-Chairman : **Dr. R.K. Singh**

Rapporteur : **Dr. Sohini Dey**

LEAD PAPERS

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B.D. Lakhchaura and V. Umapathi

IV.2 Biotechnological Approaches for Developing User Friendly & Commercially Viable Diagnostic Assays
G. Butchaiah and J. Thanislass

IV.3 Advanced Molecular Diagnostic Methodologies for Detection of Viral Diseases of Poultry
K. Dhama and M. Mahendran

IV.4 Application of different Bio-analytical techniques for Quality Control of Veterinary Biologicals
Rishendra Verma

IV.5 Application of different Bio-analytical techniques for Quality Control of Veterinary Biologicals
Rishendra Verma

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4.03 Detection of Classical Swine Fever Virus by Immunodiagnostic Tests and Nested RT-PCR
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4.04 Detection of Group A Rotavirus by Sandwich ELISA and RNA Electropherotyping
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4.31 Identification of animal species using PCR- based molecular techniques
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CONCEPTS OF DESIGNING IMMUNODIAGNOSTIC ASSAYS

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Immunological approaches to diagnostic assays have come a long way since the concept was introduced for insulin in the 1960s, and for thyroxine in the 1970s.

Immunoassays use antigens or antibodies as analytical tools and are based on the observation that in such a system, the distribution of free, as opposed to bound, analyte forms are quantitatively proportional to the analyte concentration in the system. Occasionally, assays are based on a specific binding protein in combination with antibody detection. Today, immunoassays are performed routinely, with or without sophisticated instrumentation. They range from simple point-of-care tests for use in the field or doctor's office to advanced clinical assays being run in high-throughput instruments with advanced robotics in clinical reference laboratories and blood banks. In addition to the integral role of diagnosing infectious disease, the immunoassay has revolutionized endocrinological testing, and is playing a growing role in the detection of cancer and various autoimmune diseases.

With the boom of scientific technology and global monitoring of disease, immunoassay design has become a rapidly growing field. This article will focus on the immunodiagnostic approaches of InBios International Inc. (Seattle) to global infectious diseases, primarily in humans. It will also examine how small companies deal with regulatory and marketing issues, using as examples the development of an FDA-approved enzyme-linked immunosorbent assay (ELISA) for West Nile virus and a lateral-flow rapid assay to detect visceral leishmaniasis.

The Immunoassay Concept

Because immunodiagnostics use the antigen-antibody reaction as their primary means of detection, it is necessary to review some basic principles of this reaction before discussing test design.

Antibodies are host proteins produced in response to the presence of foreign molecules (antigens) in the body. They bond to antigens to form an immune complex that signals uptake and degradation by host phagocytic immune cells. Antibodies are synthesized primarily by plasma cells, terminally differentiated cells of the B-lymphocyte lineage. They are a large family of glycoproteins that share key structural and functional features. Functionally, they can be characterized by their ability to bind both antigens and specialized cells or proteins of the immune system.

Structurally, antibodies are composed of one or more copies of a characteristic unit that can be visualized as forming a "Y". Each Y contains four polypeptides-two identical copies of a polypeptide called the "heavy chain" and two identical copies of a polypeptide called the "light chain." Antibodies are divided into five immunoglobulin (Ig) classes: IgG, IgM, IgA, IgE, and IgD. This classification is based on the number of Y-like units and the type of heavy-chain polypeptide they contain. Each Ig class plays a specific role in the immune response, with IgM production being the initial antibody response for most primary infections.

The region of an antigen that interacts with an antibody is known as an "epitope." An epitope is defined only by its interaction with the binding site of an antibody, and as such is not an intrinsic

property of any particular structure. Because antibodies can recognize relatively small regions of antigens, occasionally they can find similar epitopes on other molecules. This forms the molecular basis of a cross-reaction. However, the presence of similar epitopes does not necessarily imply a functional relationship. The binding of the antibody to the antigen depends entirely on noncovalent interactions, and the antigen-antibody complex is in equilibrium with the components in the free state.

Simply stated, the immunoassay concept is to use one analyte-either antigen or antibody-to sequester and detect the presence of the other. For example, the antigen from an infectious agent can be used in an external assay to detect the presence of antibodies produced in response to infection by that agent.

Selecting the Matrix

A number of considerations go into making a commercial immunoassay. Once a target disease has been decided on, the first development step is to define the most effective source of analyte in body fluids or tissues. Depending on the availability of analytes, assay systems have been developed using plasma, serum, whole blood, cerebrospinal fluid (CSF), sputum, urine, or solid-tissue extracts. The nature of the matrix chosen is important both for the clinical sensitivity and specificity of the assay and the nature of the disease.

Analytes may be present in one or more matrices, but at considerably different concentrations. For example, distinct assays have been designed to incorporate different matrices in the following circumstances:

- Early pregnancy testing that detects human chorionic gonadotropin using urine and blood.
- HIV antibody analysis in both serum and saliva.
- The indication of West Nile virus infection through serum testing and the diagnosis of nervous system involvement through the detection of analyte in CSF.
- The assaying of Mycobacterium tuberculosis in sputum, and of antibodies to the organism or related proteins in serum and urine.

Thus, selecting the matrix or matrices best suited to the source of analytes and presentation of the disease is an important first step of building a diagnostic assay.

The Detection Method

Since the use of radioactivity in the design of the radioimmunoassay (RIA) was first described 36 years ago, many alternate detection systems have been championed. The use of radioactive labels such as tritium (3H) and iodine (125I) and gamma or scintillation counters has largely been replaced by newer, nonradioactive methods. There are, however, some assays that require exquisite sensitivity and still use isotopes. Generally, though, the new detection agents are safer and more environmentally friendly, and have led to the development of more-advanced and high-throughput instrumentation.

Detection systems can be divided into two types, based on the test format. For rapid-type assays, analyte specific visual detection agents, such as reagents conjugated to colloidal gold or selenium

or colored latex-type particles, are used.7 These particles are sensitive, but usually not as sensitive as an enzyme-linked immunoassay. As expected, the visual and inexpensive, one-step elements of detection are essential for point-of-use test design.

For instrument-compatible assays such as ELISAs, a solid- or liquid-phase system is most desirable. In this format, various enzymes, including horseradish peroxidase, alkaline phosphatase, and beta-galactosidase can be conjugated to analyte specific reagents. Following the analyte reaction, certain enzyme activities are studied using a sensitive substrate. This can include either chromogenic-, fluorogenic-, or chemiluminescence-generating substrates, which are typically detected by light-absorbance measurements (optical density). In addition, analyte specific reagents can be coupled directly to a fluorescent, chemiluminescent, or bioluminescent tag.

This new generation of detection agents provides extremely sensitive detection systems that can be used with high-throughput instrumentation and are helpful in developing simultaneous multianalyte assays.

Selecting the Format and Method

Deciding whether to test for the presence of antigen or antibody depends largely on the target diagnosis. In screening drugs of abuse, for example, inhibition immunoassays are used. For diagnosing malarial and hepatitis B viral infections, as well as several bacterial pathogens that cause acute illness, testing for antigen in blood, rather than for antibody, is crucial. Antibody detection in blood has also been critical for visceral leishmaniasis, hepatitis C, and syphilis infection. With some diseases that elicit prolonged immunity responses, discerning between primary and secondary infections can be challenging. Cell-based assays, such as those that use T-cells for cytokine or interferon-gamma induction to diagnose latent infection, are becoming popular for diseases such as tuberculosis.8

The ELISA and the rapid, or dipstick, test currently are the two predominant formats in immunodiagnostics. ELISAs are created by coating the antigen or antibody on a suitable plastic. To complete the reaction, an enzymatic detection method with a color-forming substrate is required.

ELISAs can be either competitive or noncompetitive. The noncompetitive, or sandwich type, of ELISA is a standard tool for quantifying the antibody or antigen in a serum. One supplied analyte "captures" the complementary antigen or antibody in the serum. Then, a second labeled antibody coupled to a detection method completes the sandwich.

The West Nile Detect IgM Capture ELISA by InBios International is an example of this type of assay. To conduct the test, serum samples are applied to microtiter wells that have been coated with antihuman IgM. A West Nile-specific recombinant antigen is then applied to bind any West Nile IgM antibody complex. Finally, a West Nile antigen-specific antibody, coupled to a detection method, is used to "sandwich" the antigen and produce quantitative results.

In the competitive ELISA, a known amount of labeled analyte directly competes with the same analyte in the patient's serum. A well-designed ELISA is a prerequisite, even if the design goal is a rapid test. ELISAs help identify the correct sera (both positive control and negative control) used to optimize the subsequent lateral-flow or flow-through dipstick assay. However, while the ELISA format affords a sensitive and detailed look at analyte-sera interactions, it typically relies on large instrumentation and long reaction times, and therefore cannot truly be called a rapid test.

In rapid diagnostic tests, similar to those that InBios has developed for leishmaniasis and Chagas (T. cruzi) diseases, the antigen-antibody reaction occurs on a membrane support. These assays are conventionally called "antibody-capture assays." In this process, the antigen is attached to a solid support, and labeled antibody is allowed to bind. After the excess antibody flows upward (in a lateral-flow assay), the test is either quantitated by measuring the intensity of the test line, or yes/no information is extracted by visually confirming the test line. The latter method is suited for rapid screening and point-of-use applications.

Recent technological advances have created multiplex formats and instrumentation that enable concomitant sampling of multiple analytes. When applied to 96-well ELISAs, the multiplex format allows up to 100 distinct target immunoassays within a single assay well. In one particular multiplex system, target analytes are bound to a microsphere distinguished by a precise internal dye ratio. The appropriate instrumentation reads both a fluorescence reporter bound to the captured analyte and the microsphere itself to distinctly analyze each miniimmunoassay within a single well suspension.9 This technology seems likely both to improve specificity and broaden the spectrum of immunodiagnostics.

Monoclonal or Polyclonal Antibodies?

If a test design involves antigen capture, the next step is to produce antibody. Antibodies primarily of the IgG class are used in developing immunoassays for specific target antigens, and can be either polyclonal or monoclonal. Typically, polyclonal antibodies are developed in animal species such as sheep or goats, where large quantities can be produced. In general, polyclonal antibodies, when used to develop an immunoassay, are affinity-purified against the antigen to render them monospecific.

Monoclonal antibodies are typically developed in mice using well-established hybridoma techniques, but other species, such as rats and rabbits, have also been used. Monoclonal antibodies can be designed to hit different epitopes on the same antigen molecule, making them the antibodies of choice for use in sandwich immunoassays. They can also be designed with high affinity and specificity. In contrast, affinity-purified polyclonal antibodies would hit multiple epitopes on the antigen of interest unless the polyclonal was either developed to a specific peptide epitope or was rendered monospecific by affinity-purifying against that peptide epitope.

In the case of indirect immunoassays, where, for instance, host antibodies to a particular antigen are detected, an antibody against the specific immunoglobulin would be used in conjunction with a labeling system, thus also requiring antibody production. Again, considerations for choosing the target immunoglobulin would include analyzing the most effective and encompassing tool for diagnosis.

Choosing the Antigen

Whole-cell lysate, purified protein, and recombinant protein are all sources for antigen when an antibody capture test is being developed. Sometimes, sufficient specificity is attained using lysates or partially purified proteins. Cross-reactivity and high background levels may become a factor when using multiple analytes, and therefore the effort of creating recombinant proteins is generally rewarding, especially for future regulatory submission purposes. In addition, the cloning of particularly strong epitopes will often reduce background and increase sensitivity.

The buffer in which the antigen will remain dissolved also merits attention. Typical buffers used in rapid-test development include 10 mmol phosphate, phosphate buffer saline (PBS), borate, borate with dissolved salt, sodium acetate, and others. The selection of a buffer's pH is crucial for optimal antigenic activity and is influenced by the isoelectric point (pI) of the antigen. For some buffers, a peculiar problem arises: Newly made tests perform well and provide good sensitivity and specificity; but as they age, performance worsens.

Moreover, buffers are antigen dependent. One antigen may perform well in PBS (pH = 7.4) and may provide the desired shelf life. However, the same buffer and pH may give disastrous results with a different antigen. There is no general rule for the selection of buffer. Experimental results are the only indicators.

When an antigen is dialyzed in a given buffer and the buffer is not compatible, the antigen might precipitate out of solution. This is an early indication of an adverse antigen-buffer interaction that might produce poor antigenic activity.

Knowledge of the antigen's pI can also help researchers select the appropriate buffer. The antigen to be detected can be a circulating antibody to a specific antigen, which is the case in the Kalazar Detect rapid test for visceral leishmaniasis by InBios. In this antibody capture assay, a recombinant protein, rK39, with repeating epitopes, is immobilized on a solid phase (membrane or 96-well plate) and incubated with serum from visceral leishmaniasis patients. This is followed by incubation with goat antihuman IgG-HRP (ELISA) or labeling with colloidal gold for lateral flow.

Prototype Assays

During the development process, it is necessary to create a prototype that incorporates all of the required assay features. At this stage, sensitivity and specificity are assessed and the assay reagents fine-tuned. The resulting prototype is field tested before being committed to clinical trials.

To carry out thorough prototype testing, confirmed positive samples as well as negative controls are used to establish sensitivity and specificity data. (Inadequate specificity, for example, might necessitate looking at alternate antigens or adding epitopes.) Interlab, interassay, and intraassay reproducibility data must also be assessed to establish the robustness of the assay. The stability of kit reagents is tested at a variety of temperature and environmental conditions (e.g., humidity), and dilution ELISAs are used to accurately pinpoint antigen and conjugate concentrations (see Figure 4). Both real-time and accelerated stability are studied to determine kit shelf life. In the end, a design goal of the assay is to incorporate optimum sensitivity and shelf life without losing specificity.

Field and Clinical Studies

Clinical trials are needed to validate an assay and determine its performance characteristics in preparation for regulatory submission. Typically, trials are performed at three or more clinical sites. The locations of these sites should be pertinent to disease prevalence and the locations where the tests will be used. The purpose is to show that the test's results are reproducible at different sites and that its performance characteristics are confirmed independent of the manufacturer. Studies of the assay's specificity, sensitivity, interfering substances, and cross-reactivity are performed, as are comparisons to established reference methods. For example, among West Nile virus diagnostics, the plaque reduction neutralization test (PRNT) serves as the standard method for comparison.

Regulatory Approval

To be sold commercially in the United States, immunoassay kits must obtain regulatory approval. For assays that involve human testing, this means approval through FDA. Veterinary applications require approval from the U.S. Department of Agriculture. With regard to FDA, most of the IVDs discussed in this article would require either a premarket approval or a 510(k) clearance.

Once a test is submitted to FDA, the clinical trial results are reviewed and additional studies performed as necessary to answer specific questions. This may take anywhere from three to six months before final approval is received. As the process unfolds, marketing can continue; however, all kits and reagents must be labeled "for research use only." As part of a regulatory approval, manufacturers are open to inspection and scrutiny by FDA or other regulatory bodies to ensure product quality.

To market an assay in the European Union or European Economic Area, a similar approval process is required, resulting in the mandatory CE marking of the product. The practice of using one or more analyte specific reagents to develop a diagnostic product for use in-house is referred to as "home-brew" manufacturing and is common in less-developed parts of the world, where healthcare expenditures prohibit the widespread use of commercial kits. The quality of these tests is often quite good and they can be customized for specific needs. However, overall, they may lack the standardization and quality control assurances that accompany approved kits.

Marketing

Once regulatory approval has been obtained, marketing of the diagnostic can begin. Marketing for products from large companies is typically performed through the companies' own marketing and sales divisions. However, for small companies, such departments are typically not feasible until sales reach a certain level. Instead, small companies must rely on other methods for promoting their products. These include providing information at scientific symposia through an exhibitor's booth and presenting scientific abstracts or talks describing the performance of their products. A growing number of small diagnostics companies are also using Web sites to promote and sell their products. When marketing IVDs to foreign countries, it may be prudent to set up distributorships in the countries of interest. These distributors help speed both the importation of approved test kits and their sale to laboratories and testing sites. They can also help deal with the foreign FDA-equivalent authorities, if necessary.

Conclusion

There is an urgent need to develop reliable, user-friendly, and inexpensive diagnostic tools and vaccines for countering infectious diseases worldwide. Monitoring potential pandemics has become increasingly difficult with today's global migration patterns, and effective isolation of a disease requires quick diagnosis. Furthermore, the specificity and sensitivity of a test must be of the highest quality to be fully adaptable. The considerations for immunoassay design presented here represent an overview of the multifaceted development process.

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BIOTECHNOLOGICAL APPROACHES FOR DEVELOPING USER FRIENDLY & COMMERCIALLY VIABLE DIAGNOSTIC ASSAYS

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Laboratory tests for the detection of veterinary pathogens have traditionally relied on conventional methods. Recently, molecular diagnostic techniques such as Nucleic Acid Probes, Polymerase Chain Reaction etc., have become routine diagnostic tools in veterinary laboratories. The role of PCR has expanded and it is used for the rapid and sensitive diagnosis of diseases during out-breaks. The development of different variations of PCRs has improved the sensitivity and specificity as well as the extent of application of this technique. Under this scenario, the newer biotechnologies like transcriptomics, proteomics and nanotechnologies are fast emerging which are likely to be widely used in the future as they can improve diagnostic capabilities while reducing the time and perhaps the cost of diagnosis.

Until now, the tools of genetics have been restricted to a clinical setting, but the Human Genome Project has led to the development of substantial new technologies that are capable of defining large sets of biomarkers systematically in biological samples. The systematic approach of analysing all the products of the genome at a messenger RNA or protein level has emerged from the development of new detection technology and the availability of the genome sequence (1,2). These methodologies, all currently being used in a research setting, will generate data on multiple biomarkers that vary quantitatively very early in disease, with disease onset, with disease progression or with therapeutic response.

DNA Microarrays for Disease Diagnosis:

DNA microarrays offer the latest technological advancement for multi-gene detection and diagnosis. They were conceived originally to examine gene expression for large numbers of genes (3, 4, 5), but have also been applied to DNA sequence analysis (6), to study genes related to immune response (7), genotyping and the development of diagnostics (8, 9). In the latter context, they can be used to distinguish between DNA sequences that differ by as little as a single nucleotide polymorphism (SNP) (10). In addition, their flexibility and high throughput capabilities hold tremendous potential for pathogen detection, identification, and genotyping in molecular diagnostic laboratories. Nevertheless, an important factor in the employment of any technique is cost. High-density arrays may be very valuable in disease diagnosis (11), but at the present cost of commercial array, they are unlikely to see routine use in veterinary medicine. Fortunately, there are a number of low-cost alternatives now available both for array fabrication and detection chemistries that will overcome the cost of microarray based diagnosis.

DNA microarrays can be used to detect multiple pathogens based on differences in 16S rDNA sequences. For example, nucleic acids can be extracted from a sample and 16S rDNA sequences amplified by PCR using universal 16S primers (12). The resulting PCR products can be hybridised to an array consisting of many oligonucleotide probes, which can be designed to detect and characterize pathogens by taxonomy (e.g. Gram type) (13), by genus, or by species if sufficient discriminatory sequences are available. McCabe *et al.* (14) have constructed a similar pathogen detection array in a membrane-based, macroarray format that could be adapted to microarrays.

This type of PCR genotyping is ideal where multiple sequence differences are known to exist between conserved primer regions. Indeed, most multiplex PCR assays can be adapted for rapid detection on microarrays, although it is advantageous to limit total product length to less than 150 bp in order to enhance PCR and hybridisation efficiency. Under ideal conditions, it may be possible to multiplex ten or more PCR primer pairs for detection on a microarray. Clearly, probe sequences must be identified a priori before these types of arrays can be constructed.

Other potential applications of microarrays are the fingerprinting of bacterial isolates using very short, random hexamers (15) or by adapting expression arrays, and the identification of microbes by direct detection of 16S rRNA (16). These methods are also suitable for expression arrays. If a number of diagnostic RNA markers are identified for specific disease conditions (12), then more cost effective, low-density arrays could be designed for use in diagnostic laboratories.

Proteomics for Disease Diagnosis:

Proteins are functional molecules, and the study of proteins is of major interest to physiologists and physician scientists. The new technologies and the sequencing of the genomes of humans and other species offer opportunities to study genes and proteins on a larger scale than ever before. Because one protein may cause cascade effects and change the synthesis rate, modifications, and folding of several other proteins, large scale proteomics may yield crucial information on regulation of body functions and the mechanism of diseases. However, the proteomic technologies are still not sufficiently precise and sensitive enough to detect changes in concentrations of proteins that occur during acute physiological interventions. Moreover, proteins that are newly synthesized may get degraded immediately (fast turnover of proteins), and changes in detectable levels of concentration may not occur. Many protein functions are also altered by modifications (i.e., phosphorylation or glycation) without a change in concentration. Metabolic labeling in vivo with stable isotopes and identification of proteins whose synthesis rates change during an intervention may enable us to detect changes that could not be detected by profiling and quantification alone. Improvements in the techniques to accurately purify proteins from a mixture, quantification of samples with greater precision, and detection of the enrichment of stable isotope tracers in small sample sizes will help to advance our existing capabilities. Further advances in simultaneous detection of modification of multiple proteins in biological fluids and tissue proteins are also essential to fully understand the regulation of functions and the alterations that occur in diseases.

Profiling peptides in various pathological states from body fluids and comparison with healthy controls has already shown great promise in diagnosis, early detection, and monitoring prognosis of many diseases. For example, serum from a person infected with a mycobacterial pathogen has a unique pattern that can be identified using specifically designed biochip and analysis software. This type of technology may be useful for identifying animals infected with agents that do not induce predictable serologic reactions, such as bovine tuberculosis (17). The technological sophistication in multidimensional chromatography in conjunction with tandem mass spectrometry and antibody-based protein chips provides substantial technological support for this line of clinical research.

Nanotechnology Based Diagnostics:

Under Nanotechnology, the development and use of nanoscale analytical tools are the most promising areas of immediate benefit. Many of the efforts in developing nanoscale in vitro or

ex vivo measurement and molecular detection systems rely on the methods being developed to construct nanoscale electronic circuits. For example, 1–2 nm wide, boron-doped silicon nanowires laid down on a silicon grid can be coated with antigens to provide real-time detection and titering of antibodies (18). Antibody binding to immobilized antigen produces an immediate, measurable change in conductance at antibody concentrations below 10 nM. In the same study, nanowires derivatized with the calcium-binding protein calmodulin provided real-time measurements of calcium ions at physiologic levels. More recently, investigators have developed methods for chemically modifying lithographically etched silicon nanostructures to enable attachment of a wide range of molecules as the first step for creating versatile, chip-based biosensors (19). Silicon-based arrays made of antibody-conjugated nanowire field effect transistors have also been multiplexed to simultaneously detect single copies of multiple viruses (20). Functionalized carbon nanotubes can also function as highly specific electronic biosensors (21).

Individual nanoscale particles can also provide remarkable analytical sensitivity in a variety of in vitro assays, in real time and without the use of radioisotopes. Semiconducting quantum dots labeled with tumor marker antibodies, such as anti-Her-2, can greatly reduce the time needed to assess biopsy tissue by eliminating most of the steps needed to prepare a sample for analysis. The labeled quantum dots can simply be added to the biopsy tissue and the results viewed using fluorescence microscope (22). Moreover, by controlling the diameter of the particle during synthesis, quantum dots can be engineered to fluoresce at wavelengths ranging from UV to NIR. It is therefore possible to create multiplexed assays using differently colored quantum dots, each labeled with a different substrate (23). Similarly, gold nanoshells, whose optical properties also depend in a predictable manner on size, have also been derivatized with a variety of polymers and biomolecules. These conjugates have been used to detect picogram-per-milliliter quantities of antibodies and other biomolecules in whole blood. The time required for such quantitative measurements could be 30 min (24).

Non-PCR based Nucleic Acid Amplification Methods:

New methods of nucleic acid amplification have been developed and may eventually be used for veterinary diagnostics. Examples of these methods include the rolling circle amplification technique (25) and direct signal amplification systems. These techniques are currently being used in human diagnostics for the detection of human cytomegalovirus and human immunodeficiency viruses; veterinary applications are currently being developed.

Fluorescent in situ hybridisation (FISH) is a technique that can localise nucleic acid sequences within cellular material. Peptide nucleic acids are molecules in which the sugar backbone has been replaced by a peptide backbone. These molecules are perfect mimics of DNA with high affinity for hybridisation that can be used to improve FISH techniques (26).

A new proprietary, conformationally restricted oligonucleotide known as 'locked nucleic acid' shows promise for hybridisation assays. New techniques utilising locked nucleic acids can better discriminate between correct and incorrect DNA/RNA target sequences and their use looks to be increasing in many applications where oligonucleotide probes are used.

Nucleic acid sequence-based amplification (NASBA) is a promising gene amplification method. This isothermal technique is comprised of a two-step process whereby there is an initial enzymatic amplification of the nucleic acid targets followed by detection of the generated amplicons. The entire NASBA process is conducted at a single temperature, thereby eliminating the need for

a thermocycler. The use of this technique has been shown to detect avian and human influenza viruses (27, 28).

Biotechnology for the Improvement of Immunoassay:

New biotechnological methods such as the cloning of genes, over expression of immunogens, use of expression vectors and peptide synthesis have made possible the production of specific proteins or peptides that serve as target antigens or positive control reagents in existing and newly-developed immunoassays. The use of these improved antigens can increase the specificity or sensitivity of immunoassays by providing a more defined target for binding antibody and can reduce serial-to-serial (lot-to-lot) variation of test kit performance as a result of using a more homogenous and well-defined antigen to capture antibodies (29). Molecular techniques also provide opportunities to improve antigens that are used to induce polyclonal or monoclonal antibody production, select monoclonal antibodies that recognise specific target epitopes and purify antisera for specific diagnostic purposes. Use of synthetic peptides for use as positive controls in these assays provides an opportunity to include a specific positive control without the risks involved in producing killed agents. These techniques have been predominantly applied to the development or improvement of ELISAs and can be used to develop highly sensitive screening assays and highly specific confirmatory assays.

The cloning and expression of specific proteins produced by a pathogen have enabled the development of assays that can differentiate vaccinated from non-vaccinated (infected) animals. This can be accomplished by expressing a single immunogen in a vectored vaccine, or by deleting the expression of a single immunogen in a vaccine, followed by the development of a complementary serological assay(30).

The biotechnological techniques discussed above have the potential to improve the veterinary diagnostics in terms of economics, efficiency, scope, sensitivity and specificity.

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ADVANCED MOLECULAR DIAGNOSTIC METHODOLOGIES FOR DETECTION OF VIRAL DISEASES OF POULTRY

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INTRODUCTION

The poultry production, all over the world and with particular mention to India, is making rapid progress towards achieving the role of a profitable enterprise for backyard growers as well as large-scale production units. The exploiting of technological advancements in the field of science and technology and more precisely, molecular biology and biotechnology has primarily helped this revolutionary trend in poultry sector. The spotlight has now been more prominently shifted towards the proper management of poultry diseases so as to curb the losses incurred due to various viral diseases in poultry flocks. Infectious bursal disease, Infectious bronchitis, Chicken infectious anemia, Hydropericardium syndrome, Egg drop syndrome etc. has repeatedly created a mayhem among poultry growers. At the same time, exotic diseases like avian influenza have recently thrown a threat with a detrimental effect on the poultry industry and the human population as well. All this has increased the significance of rapid and specific detection of viral pathogens and finding novel alternatives for their effective control. Laboratory diagnosis for poultry pathogens have traditionally relied on isolation by cell culture or detection of virus specific antibodies using a variety of techniques such as agar gel immunodiffusion, enzyme-linked immunosorbent assay, fluoresent antibody test and neutralization tests. In comparison, the development of molecular biology has contributed to the upbringing of highly sensitive and rapid diagnostic approaches and effective control measures for combating the avian viral diseases. In the past fifteen years, poultry diagnosticians have incorporated new molecular techniques such as the polymerase chain reaction and have improved conventional immunodiagnostic techniques through the use of recombinant antigens and monoclonal antibodies. Although conventional diagnostic assays are still used routinely, the molecular techniques have broadened the scope of poultry diagnostics and are now powerful tools that assist in a comprehensive analysis of viral pathogens. The advancements in new high-throughput technologies arising from knowledge of microbial genomics is enabling the analysis of the genome and proteome and is offering the opportunity to gain a better understanding of the molecular pathways of various pathogens, apart from elucidating the host immune system and host-pathogen interactions. Utilizing the new generation biotechnological techniques and tools, it is expected that in near future the myriad poultry pathogens can be effectively wiped out for the well being of sustainable poultry production systems.

MOLECULAR DIAGNOSTIC TECHNIQUES

The application of nucleic acid-based diagnostics for poultry disease diagnosis has increased exponentially in the recent years and is assisting in the rapid diagnosis of many infectious agents. These techniques have redefined the diagnostic scenario and have improved the information available for disease control programmes. Conventional detection methods are perceived to be cumbersome and require in vivo systems. With the advent of molecular techniques, the diagnosis of the viral diseases has become more refined and reliable. The first of the molecular biological methods that was introduced in diagnostics was nucleic acid hybridization technique. But in the

recent years, polymerase chain reaction (PCR) and allied techniques along with genomic sequencing has over taken it, and has become a driving force for the development of rapid, sensitive and specific assays capable of viral genomic detection. They are widely used because of being highly sensitive and can be performed rapidly in a cost effective manner. Several different methods including PCR, real-time PCR, nucleic acid sequence-based amplification, biosensors, microarray and other techniques are described further.

Polymerase chain reaction (PCR): The molecular technique with the widest variety of application in poultry diagnostics is PCR. The strength of this technique is its ability to make millions of copies of a target DNA, enabling the desired target to be readily detected by techniques such as electrophoresis. The role of PCR has expanded and it is now being used for the rapid and large-scale diagnosis of viral diseases of domestic birds. Viral genes of pathogens are being amplified and detected from the samples using specific oligonucleotide primers. PCR have proven to be specific and sensitive method for the detection of egg drop syndrome virus, chicken infectious anemia virus, hydropericardium syndrome virus and Marek's disease virus. Similarly, for the detection of RNA viruses, reverse transcription PCR (RT-PCR) is employed. This assay initially makes a complementary DNA copy of the original viral RNA before final amplification. The technique is more sensitive than the traditional Northern blot method of RNA detection. By RT-PCR, it is possible to identify various viral pathogens of poultry such as Newcastle disease virus, infectious bursal disease virus, infectious bronchitis virus, avian leucosis virus, avian encephalomyelitis and avian influenza viruses.

Nested PCR: This strategy is based on the application of a second set of primer targeting a shorter area within the initial amplicon, which is obtained from PCR. Using this approach the sensitivity of the PCR increases as it generates two amplified products for a confirmation purpose. A disadvantage of the technique is the increased risk of cross-contamination due to the opening of PCR tubes to add an additional set of primers. Nested PCR has been employed for the specific and sensitive detection of Newcastle disease virus, ALV subgroup J viruses, infectious bronchitis virus and chicken infectious anemia virus.

Quantitative PCR (Q-PCR): This assay has been used to quantify the viral genome in clinical samples. It is performed by utilizing a competitive procedure, which involves a competitor DNA that differs from viral DNA of interest by having a small insertion. The competitor DNA acts as an internal standard for the estimation of viral DNA in the unknown sample. The amount of viral DNA in the sample is quantitated by co-amplification in the presence of known amount of competitor DNA. When the amount of competitor DNA is equal to the amount of viral DNA in a sample, there is equal amplification of the competitor and viral DNA. Thus, viral DNA in unknown sample can be quantitated. The assay has been found useful in quantifying Marek's disease virus (MDV). It can be reliably used for the quantification of MDV vaccine virus and also for monitoring vaccine virus levels in commercial chicks. In real time quantification, the viral genome load of MDV is assessed by using fluorescein tagged primers and an amplicon of chicken IFN-? as an internal standard. The utility of Q-PCR has also been extended to assess the amount of IB vaccine virus applied in hatcheries or to assess the IBD virus production in cell cultures.

Real-time PCR: The latest improvements in the standard PCR technique have paved way for the evolution of real-time detection. This technique involves the analysis of genome using fluorogenic probes that releases fluorescent signals during amplification. This has eliminated the need for

post-PCR screening by electrophoresis and allows confirmation of a virus within minutes, which is crucial for rapid implementation of control measures, especially in the event of an outbreak. It is a rapid and cost-effective diagnostic tool primarily for avian influenza surveillance and monitoring programs. It can be adapted for use in the field conditions through the use of portable thermocyclers and this approach may help in rapid decision-making. The real-time PCR, apart from detection, is also used extensively for the genotyping and phylogenetic analysis of viral pathogens. Many workers have reported the utility of real-time RT-PCR as diagnostic test for economically important viral diseases like infectious bronchitis and Newcastle disease virus NDV, using SYBR green or Taqman detection methods. The technique has also been exploited for the qualitative and quantitative detection of avian influenza and IBD. A real-time RT-PCR (RRT-PCR) assay utilizing light upon extension fluorogenic primer (LUX RT-PCR) has been recently developed for the rapid and efficient detection of avian influenza viruses. RRT-PCR has also been developed which detects influenza virus A and B types or facilitating specific identification of influenza H5 subtypes. Rapid pathotyping of the influenza subtypes is crucial during outbreaks and this can be performed using such assays, based on the analysis of the HA gene cleavage site.

Multiplex-PCR: In this technique, two or more primer pairs specific for different target sequences of different viral pathogens are included in the same reaction. The co-amplification of the several targets is more cost effective and makes differential diagnosis much easier in cases of multiple pathogens responsible for disease etiology. This technique has been utilized in the detection of viral pathogens involving avian influenza virus, Newcastle disease virus, Marek's disease virus and chicken infectious anemia virus. Aside to these, reverse transcription and PCR can be carried out with a mixture of primers specific for influenza viruses of type A, subtype H5 and N1 in a single reaction system under identical conditions, which could be valuable in the rapid detection of H5N1 influenza viruses in clinical cases. A single-step multiplex RT-PCR may also be developed for differentially detecting highly pathogenic H5 and H7 viruses.

PCR-ELISA: The sensitivity of enzyme linked immunosorbent assay (ELISA) and specificity of PCR can be combined and utilized for the detection of viral genome using PCR-ELISA. A 'probe' designed to bind to the central region of PCR product is used to facilitate detection by ELISA. This oligonucleotide probe will help in binding to the streptavidin coated ELISA plates and the amplified product is later detected using ELISA. PCR-ELISA has been used for detection of avian influenza and Newcastle disease viruses. Recently, an H9-based RT-PCR-ELISA was found highly sensitive when compared to virus isolation method in detecting H9N2 avian influenza virus from chickens.

PCR-RFLP: The technique of PCR-Restriction fragment length polymorphism (RFLP) helps in analyzing the molecular epidemiology of a disease and determines the origin of infection and involvement of different strains or types of viruses. It is performed by digesting the PCR products obtained from viral gene amplification, with restriction endonucleases (RE), which cuts only specific DNA sequences recognized by the enzymes. Alterations due to mutations in the DNA sequence changes the RE sites where these enzymes cleave the DNA, thus helping in differentiating viruses belonging to distant lineages. PCR-RFLP has been employed for characterization of EDS-76 virus isolates. This technique has also been employed for differentiation of isolates of NDV, ALV, IBDV and IBV. PCR-RFLP of four genome regions of ILTV has been recently utilized to characterize 25 isolates, which classified them into nine groups.

Gene Sequencing and Phylogenetic Analysis: Gene sequencing followed by phylogenetic analy-

sis, using bioinformatics softwares like 'Gene Tool' and 'DNASTAR', has been done for a number of avian viruses for molecular characterization and to analyze the role of specific genes coding for proteins that contributes to pathogenicity. Advances in DNA sequencing technology have made it possible to sequence whole genome or some of the specific genes of avian viruses. Access to the DNA sequences through databases like GenBank as well as EMBL is helping researchers to develop sequence specific primers and gene probes apart form helping to analyze the viruses at their genomic level. Whole genome sequencing has already been done for Marek's disease virus and Fowlpox viruses and sequencing of important genes of NDV, AIV, ALV and EDS-76 virus has been done for analyzing their epidemiological status and also to assess the genomic relationship or isolate variations. Nucleotide sequences of the VP2 hypervariable region of IBDVs isolated in Africa have proved that the isolates diverged into two genotypes within the very virulent type. The phylogenetic relation of avian influenza viruses based on HA gene sequences has been performed which brought to light that all AIVs that have multiple basic amino acids at the hemagglutinin-connecting peptide shows highly pathogenic nature and the H5N1 viruses have been classified into different clades based on such sequence information, thus identifying potential candidates for a pandemic.

Nucleic acid sequence based amplification (NASBA): It is a promising gene amplification method especially suitable for viruses with RNA genomes. This isothermal technique is comprised of a two-step process of initial enzymatic amplification of the nucleic acid targets followed by visualization of the generated amplicons using chemiluminescent detection system. NASBA allows the rapid amplification of specific regions of nucleic acid obtained from a diverse range of sources. The NASBA technique is a rapid and sensitive method of detection for pathogenic influenza viruses. A NASBA method has been developed to detect a portion of the haemagglutinin gene of AIV subtypes H5 and H7 irrespective of lineage. Recently, NASBA has also been reported to be a potential method to rapidly and reliably detect NDV isolates.

DNA Microarray: Microarray technology, originally developed for mapping of genes, is being used to detect a wide variety of pathogens of veterinary significance. In this technique, specific oligonucleotides are bound to small solid supports such as glass slides, silicon chips or nylon membranes. Extracted DNA or complementary DNA is labeled with a fluorescent dye and then hybridized with the microarray. Specific patterns of fluorescence are detected by a microarray reader, which allows the identification of specific gene sequences or expression of a particular gene during different stages of virus multiplication. This technology also has the potential to identify the presence of viruses at the serotype or subspecies level, or to differentiate agents that cause similar clinical signs. Simultaneous testing for detection of infectious pathogens that cause similar symptoms is invaluable for treatment, outbreak prevention, and efficient use of antibiotic and antiviral agents. In addition, such testing may provide information regarding possible co-infections or secondary infections, such as virally induced bacterial infections. In avian species, DNA microarrays containing approximately 1200 genes were found to effectively detect differences in gene expression between the inbred chicken lines of uninfected and MDV-infected peripheral blood lymphocytes. Another microarray has been developed for the reliable detection of H5, H7 and H9 subtypes of AIV in which the cDNAs encoding approximately 500 bp influenza virus gene fragments obtained by RT-PCR technique were spotted on a glass-bound microarray. Besides, a microarray has been recently developed to provide individual detection sensitivity for more than 26 respiratory pathogens while still retaining the ability to detect and differentiate between close genetic neighbors.

Biosensors: The approach of biosensing, to detect either the viral pathogen or virus-specific antibodies, has been considered as recently developed technique. Biosensor assays involve the use of a receptor (for eg. antibody) for the target pathogen or a disease-specific antibody together with a transducer that converts the antigen antibody interaction into a measurable signal. For this purpose, transducer technologies like electrochemical sensing, interferometry, resonance and fluorimetry are applied. Fluorescence polarization technology, available for the detection of pathogens, relies on the use of fluorescence to label antigens or antibodies in a standard preparation and variation in the spin of the labeled molecule is determined using a specialized fluorimeter. This technology offers a rapid detection method, taking only minutes to analyse each sample and could be applied for the detection of poultry pathogens. Similarly, fluorimetry is being adapted to light fibre-technology biosensors. Also, recombinant antigen based surface plasmon resonance biosensor can be developed to detect anti-viral antibodies in birds. Besides, a multi-analyte array biosensor (MAAB) could be a useful tool for the rapid analysis of multiple analytes simultaneously, which could favor identification of variants or serotypes of avian viruses.

Recombinant DNA technology and novel diagnostics: This technology assists in the mass production of recombinant antigens that could be exploited in conventional techniques such as ELISA. Recombinant technology, using either the prokaryotic or eukaryotic system, has the ability to generate large amounts of protein molecules that can be commercially exploited for developing novel and rapid sero-diagnostic tools. Besides, this technique has found its application in developing subunit vaccines. The recombinant technology-based generation of antigens has considerably lessened the need for the bulk cultivation of viral pathogens using cell culture systems, which is time consuming as well as cumbersome. Moreover, in case of CIAV, the growth in cell lines like MDCC-MSB1 requires stringent conditions and poses several problems while culturing. The recombinant DNA technology can overcome this difficulty by supplying CIAV viral antigens in comparatively cheaper and a less strenuous manner. Further, serotype-determining regions of the different viral proteins can be expressed using this technique to develop detection kits to analyze variant viruses. Likewise, the specific proteins of pathogens like AIV can be used to develop assays that can differentiate vaccinated from non-vaccinated (infected) animals (DIVA).

Nanotechnology and its application in disease diagnosis: Nanotechnology is the creation and utilization of materials, devices and systems through the control of matter at nanometric scale. This novel technology delivers products and processes at nanoscale dimensions through the characterization and manipulation of matter at atomic levels. Nanotechnology operates at the same scale as a virus and holds the potential for real-time detection and eradication of diseases. The development of the rapid detection test initially uses the specific antibodies that detect viral antigens in the sample using nanoparticle platform. These nanoparticle-labelled antibodies can then be used in various assays to identify specific pathogens. The current laboratory-based ELISA could be modified into a highly rapid detection test based on the gold nanoparticle clusters and by using rapid colorimetric detection. Another advantage of this technology is the potential to analyse a sample for an array of infectious agents on a single chip. Applications also include the identification of specific strains or serotypes of viruses, or the differentiation of diseases caused by viruses manifesting similar clinical signs. Examples of nanoparticle technology include the use of gold nanoparticles, nanobarcodes, quantum dots and nanoparticle probes. Considerable scope exist in the application of nanotechnology in the field of avian viral disease diagnosis, for which there is need for targeted research in this promising area so that in coming years, the avian health care sector could be revolutionized.

Conclusion

A significant leap in the advancement in the field of genomic detection utilizing the advanced molecular biology tools has provided the ways and means for rapid detection of avian viral pathogens. Rapid detection and characterization of a viral pathogen is of paramount importance in developing prophylactic strategies for poultry diseases. The versatility of PCR technique and the introduction of advanced research amenities like real time-PCR, quantitative PCR, genomic profiling etc. have catapulted the avian viral disease diagnostic sector. Nucleic acid detection assays based on PCR amplification has been used in a prolific and reliable manner to rapidly diagnose and characterize avian viral pathogens. The genetic characterization of economically important viral pathogens like NDV, AIV, CIAV, IBDV and IBV has been facilitated by PCR-RFLP as well as type-specific PCR's. As the poultry viruses, especially IBV has the ability to continually generate variant strains, much more targeted genomic anlaysis have to be employed. Pathobiological interactions of various avian viruses with host tissues can be analyzed by employing latest techniques such as microarray technology, thus facilitating the identification of genes expressed during various stages of viral infections. Further, the use of biosensor technique and nanotechnolgy for the identification of viral pathogens has been looked upon as detection tools in the near future. Besides, the technology of genetic engineering has paved way for generation of advanced and much more efficacious vaccines that targets the macromolecules of viral entities. Also, more research tools are to be standardized for their fruitful application in detection, pathogenic studies and genomic profiling of avian viruses. Based on the promising molecular research works and biotechnological approaches carried out on avian viruses, one can be optimistic that soon we may equip ourselves to effectively wipe out such harmful pathogens that cause devastating outcomes to the poultry sector.

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APPLICATION OF DIFFERENT BIO-ANALYTICAL TECHNIQUES FOR QUALITY CONTROL OF VETERINARY BIOLOGICALS

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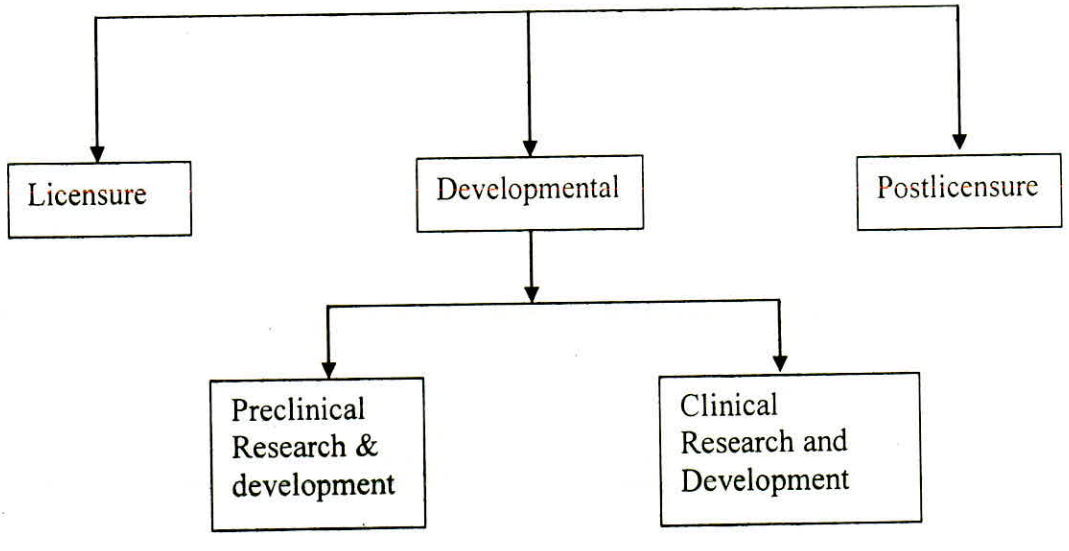
Vaccinology is the science of vaccine development. Vaccination is a deliberate attempt to immunize human or animal. It goes over 200 years ago, when an English Physician Edward Jenner observed that milk maids who contracted a mild viral disease called cow pox were rarely suffered from smallpox. The term "vaccine" is derived from a Latin word "Vacca", means cow- a reflection on Jenner's pioneering studies using cowpox vaccinia virus to prevent human smallpox (variola). Vaccines take advantage of using relatively harmless antigens (immunogen) to evoke protective immunity that resists infection and or disease pathogenesis. There are many types of vaccines including inactivated (killed) vaccines using killed or inactivated microbes, attenuated microbes, inactivated toxins (Toxoid), purified proteins or polysaccharides. There are also new strategies that may be used in vaccinology like DNA vaccines, edible vaccines etc.

In the present and recent scenario, the majority of emerging infectious diseases are of zoonotic origin i.e. transmissible between humans and animals causing infection in both species. If we look to past 10 years, the science of vaccinology is to respond to SARS-associated coronavirus identified in some domestic and wildlife specie, Nipah virus from bats via pigs, influenza viruses from birds and the West Nile virus from birds via mosquitoes. There are also already existing zoonotic infections and problem of antimicrobial resistant organisms which pose a challenge to develop strategies to address this situation. In this context, the development of effective vaccines represents one of the most promising approaches for providing cost-effective interventions against zoonotic and animal infectious diseases. Animal models have contributed to the considerable progress of our understanding of the mechanism of immunity and disease pathogensis associated with infectious agents by providing identification of vaccine candidate antigens, and in demonstrating proof-of-principle vaccine strategies. It is well known fact that vaccines can be effective strategy to



control infectious diseases, and in this context veterinary medicine is at the interface between animal and human health (rtripp@vetuga.edu).

The Quality Assurance of any vaccine is of utmost importance. The regulation of vaccines can be divided into three stages

Preclinical research and development is carried out in the laboratory and often uses animals, but never humans. Preclinical and laboratory research data include details of the development and production of a vaccine together with reports of control testing. This is done to justify adequately subsequent studies in humans.

Clinical trials in humans are classified into three phases: phase I, phase II and phase III. In certain countries forma regulatory approval is required in order to undertake clinical phase II and phase III studies.

Phase I : initial testing of vaccine is carried out in small numbers of human subjects, to test the properties of a vaccine, level of toxicity, metabolic and pharmacological effects. Phase I studies are primarily concerned with safety.

Phase II : studies involve large number of subjects and are intended to obtain information about a vaccine's ability to produce its desired effect (immunogenicity) in the target population and general safety.

Phase III : it is an extensive study to fully assess protective efficacy and safety of a vaccine. By the beginning of phase III stage of development, a vaccine should have been fully characterized and batch release testing procedures established.

Phase IV : Post-market surveillance (PMS) should be carried ut after licensure, when a vaccine is already in use. The objectives are to detect adverse reactions and to monitor efficacy/ effectiveness.

Stability of Vaccines: The stability of vaccines has a major impact on the success of immunization programmes worldwide. Stability is the ability of a vaccine to retain its chemical, physical, microbiological and biological properties within specified limits throughout its shell-life. In the 1980s and the beginning of the 1990s, a major WHO focus was on thermostability testing as measured by potency assays, as part of lot release. The temperature sensitivity of vaccine characteristics, particularly potency, led to the development of storage and cold chain requirements for all vaccines.

Tests incorporated into vaccine stability studies used to determine vaccine characteristics, including biological activity (e.g. potency, antigen content, specific toxicity etc) are performed prior to or after vaccine exposure to:

1. recommended storage temperature (real time real storage condition studies)

2. elevated temperatures (accelerated stability studies) e.g. thermostability

The vaccine should be manufactured complying to current Good Manufacturing practice (GMP) and Good Laboratory Practice (GLP). Vaccines are tested as prescribed test protocols provided in India in Indian Pharmacopoeia (2007) and in general the following tests are applied to test a vaccine depending upon the type of vaccine.

Sterility: The *tests for sterility* are designed to reveal the presence of microorganisms in the samples used in the tests.

Culture media: Media may be prepared from ingredients or dehydrated mixtures may be used provided that when reconstituted as directed by the manufacturer, they have growth pill assure anaerobic conditionsromoting properties. The following media are used:

1. Fluid thioglycollate medium: use it by incubating it at 30^0 to 35^0 under aerobic conditions, observe for 7 days
2. Alternate thioglycollate medium: use it by incubating at 30^0 to 35^0 observe for 7 days
3. Soyabean-casein digest medium: Use by incubating at 20^0 to 25^0 under aerobic conditions
4. Sabouraud's Dextrose Agar for fungi

Safety : in animal models or homologous hosts

Titre of virus: Using cell culture as per the vaccine

Estimation of 146s antigen: in FMD vaccine manufacture

Moisture: The moisture content is measured in freeze dried vaccines

Potency: Most of veterinary vaccines in India are tested for their potency in homologous test by

1. Direct challenge infection
2. Determination of HA/HI titre
3. Gel precipitin test
4. Protective Dose (PD_{50})
5. Innocuity in some vaccines like FMD
6. Toxin and antitoxin titration

WILDLIFE AND EMERGING ZOONOSES

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Abstract

Zoonotic pathogens are the most significant cause of EIDs affecting humans, both in the proportion of EIDs that they cause and in the impact that they have. Some 1,415 species of infectious organisms are known to be pathogenic to people, with 61% of them being zoonotic. Emerging infectious diseases have a major effect on human health and can create tremendous economic losses. Emerging and reemerging infectious diseases have received increasing attention since the end of the 20th century. Of the emerging pathogens, 75% are zoonotic ? mainly of viral origin and likely to be vector-borne. Zoonotic pathogens are twice more likely to be associated with emerging diseases than are non-zoonotic pathogens. Infectious pathogens of wildlife affect not only human health and agricultural production but also wildlife-based economies and wildlife conservation. More important, zoonotic pathogens cause a series of EIDs with high case fatality rates and no reliable cure, vaccine, or therapy. Thus, zoonoses with a wildlife reservoir represent a major public health problem, affecting all continents. Hundreds of pathogens and many different transmission modes are involved, and many factors influence the epidemiology of the various zoonoses. Zoonotic pathogens that infect domestic animals and wildlife hosts are more likely to emerge and hence the importance and recognition of wildlife as a reservoir of zoonoses are increasing. This paper discusses about the role of wildlife as reservoirs, increasing emergence of zoonoses, factors for emergence, and measures for prevention and control.

Preamble

Emerging infectious diseases have a major effect on human health and can create tremendous economic losses. Emerging and reemerging infectious diseases have received increasing attention since the end of the 20th century. An estimated 75% of emerging infectious diseases are zoonotic, mainly of viral origin, and likely to be vector-borne (Taylor *et al.*, 2001). Animals, particularly wild animals, are thought to be the source of >70% of all emerging infections (Kuiken *et al.*, 2005). Infectious pathogens of wildlife affect not only human health and agricultural production but also wildlife-based economies and wildlife conservation. Zoonotic pathogens that infect domestic animals and wildlife hosts are more likely to emerge (Cleaveland *et al.*, 2001). Furthermore, our quest for close contact with wild animals and for exotic pets puts us at risk for exposure to zoonoses.

Most emerging infectious diseases are zoonotic; wildlife constitutes a large and often unknown reservoir. Wildlife can also be a source for reemergence of previously controlled zoonoses. Although the discovery of such zoonoses is often related to better diagnostic tools, the leading causes of their emergence are human behavior and modifications to natural habitats (expansion of human populations and their encroachment on wildlife habitat), changes in agricultural practices, and globalization of trade. However, other factors include wildlife trade and translocation, live animal and bushmeat markets, consumption of exotic foods, development of ecotourism, access

to petting zoos, and ownership of exotic pets. To reduce risk for emerging zoonoses, the public should be educated about the risks associated with wildlife, bushmeat, and exotic pet trades; and proper surveillance systems should be implemented.

Historical Aspects

Zoonoses have affected human health throughout times, and wildlife has always played a role. For example, bubonic plague, a bacterial disease for which rats and fleas play a central role in transmission, has caused substantial illness and death around the world since ancient times. A possible epidemic of bubonic plague was described in the Old Testament, in the First Book of Samuel. The so called Black Death emerged in the 14th century and caused vast losses throughout Asia, Africa, and Europe. The epidemic, which originated in the Far East, killed approximately one third of Europe's population. However, bubonic plague still occurs in Asia, Africa, and the Americas, and the World Health Organization annually reports 1,000-3,000 cases. In the western United States, acquisition of plague in humans is linked to companion animals infested with Yersinia pestis-carrying fleas in areas of endemic sylvatic disease. Rabies was described in Mesopotamia, in hunting dogs, as early as 2,300 BC. Recognizable descriptions of rabies

can also be traced back to early Chinese, Egyptian, Greek, and Roman records. In Europe in the medieval age, rabies occurred in both domestic animals and wildlife. Rabid foxes, wolves, badgers, and bears have been described in the literature as well as in figurative art. Ancient accounts and modern hypotheses suggest that Alexander the Great, who died in Babylon in 323 BC, died of encephalitis caused by West Nile virus, a virus that has a wild bird reservoir. Marr and Calisher reported that as Alexander entered Babylon, a flock of ravens exhibiting unusual behavior died at his feet. In 1999, West Nile virus was introduced into the United States, where it caused the serious epizootic in birds with a spillover of infections to humans and equines (reviewed by Kruse *et al.*, 2004).

Wildlife as a reservoir for zoonoses

Zoonoses with a wildlife reservoir represent a major public health problem, affecting all continents. Hundreds of pathogens and many different transmission modes are involved, and many factors influence the epidemiology of the various zoonoses. The importance and recognition of wildlife as a reservoir of zoonoses are increasing (Kruse *et al.*, 2004).

Transmission Modes

Zoonoses with a wildlife reservoir represent a large spectrum of transmission modes. Several zoonotic agents can be directly transmitted from wildlife to humans, e.g., Francisella tularensis, the causative agent of tularemia, can be transmitted by skin contact with an infested, diseased, or dead hare or rodent. By contrast, rabies virus is transmitted by bite (saliva) from a rabid animal. Hantaviruses are spread from rodents to humans by aerosols in dust from rodent excreta. Zoonotic agents can also be spread from wildlife to humans indirectly by contaminated food and water, for example Salmonella spp. and Leptospira spp. Many zoonoses with a wildlife origin are spread through insect vectors. For example, mosquitoes are well known vectors of several wildlife zoonoses, such as Rift Valley fever, equine encephalitis, and Japanese encephalitis. Y. pestis can be spread by fleas, Bacillus anthracis spores by flies, and Leishmania by sand-flies, whereas

ticks are essential in the spread of Borrelia burgdorferi and Ehrlichia/Anaplasma. A good example

of a zoonotic agent with many different transmission modes is F. tularensis. Rodents and hares constitute the main sources of infections, and hunters are at

particular risk of acquiring the disease. The transmission mode also affects the clinical manifestation in humans. The agent can be transmitted by direct contact through the handling of an infected carcass and through tick or mosquito bites, which cause initial skin symptoms such as ulcers. Infection may also occur after eating insufficiently cooked meat from an infected animal or contaminated drinking water, causing symptoms from the digestive tract, and by inhalation of contaminated dust, causing a pneumonialike illness. Salmonella spp. can also be spread from wildlife to humans in different ways (reviewed by Kruse *et al.*, 2004).

Reptile-associated salmonellosis is a well-described phenomenon, especially among children. The increasing popularity of keeping reptiles and other exotic animals as pets presents a public health problem, as such animals are commonly carriers of Salmonella and thereby can infect humans directly or indirectly. In Norway, special types of Salmonella enterica subsp. Enterica serovar Typhimurium (S. Typhimurium) occur endemically in hedgehogs and wild passerine birds, causing sporadic cases and small outbreaks in humans. In 1987, a nationwide outbreak of S. Typhimurium infections was traced to chocolate bars that had been contaminated by wild birds in the factory. In 1999, a waterborne outbreak of S. Typhimurium infections was linked to a dead seagull that had contaminated a reservoir water source from which the water was used untreated (8-10). B. anthracis, the etiologic agent of anthrax, primarily a disease of herbivores, can also be transmitted from wildlife to humans by various modes. The spores formed by the bacteria are very resistant and have been found to remain dormant and viable in nature for >100 years (11). Anthrax is spread by food and water contamination or by the spread of spores by flies, vultures, and other scavengers. Humans can be infected by eating meat from infected carcasses or drinking contaminated water, through the skin by contact with infected material or by insect bites, and through the lungs by inhaling spores. Although livestock anthrax is declining in many parts of the world, the disease remains enzootic in many national parks, for example, in southern Africa and North America. Anthrax in wildlife represents a persistent risk for surrounding livestock and public health (12) (reviewed by Kruse *et al.*, 2004).

Factors Influencing the Epidemiology of Zoonoses with a Wildlife Reservoir

Leading factors for emergence of zoonoses are unbalanced and selective forest exploitation and aggressive agricultural development associated with an exponential increase in the bushmeat trade (Wolfe *et al.*, 2005). Similarly, the increase of ecotourism, often in primitive settings with limited hygiene, can be associated with the acquisition of zoonotic agents. The ecologic changes influencing the epidemiology of zoonoses with a wildlife reservoir can be of natural or anthropogenic origin. These include, but are not limited to, human population expansion and encroachment, reforestation and other habitat changes, pollution, and climatic changes. The spirochete Borrelia burgdorferi, which causes Lyme borreliosis, has its main reservoir among small rodents and deer and uses various Ixodes species as vectors (13). Lyme borreliosis was first recognized in Lyme, Connecticut, in 1975, and since then, an increasing number of cases have been reported in North America, Europe, and Asia. The increasing incidence of Lyme borreliosis in the northeastern United States in recent years can be explained by reforestation that has favored transmission of the disease through increased populations of white-tailed deer and deer mice and abundance of the tick vector, Ixodes scapularis. Wild rodents also constitute a reservoir of hantaviruses (14). The viruses are shed in urine, droppings, and saliva, and humans are mainly infected aerogenically

by inhaling aerosols containing the virus. Precipitation, habitat structure, and food availability are critical environmental factors that affect rodent population dynamics as well as viral transmission between animals and subsequently the incidence of human infection. The deer mouse is a reservoir host for Sin Nombre hantavirus, which causes hantavirus pulmonary syndrome in the southwestern United States. Because of climatic changes with increased rainfall in recent years, host abundance, and thereby spread of the pathogen, has increased, with subsequent transmission to humans. The movement of pathogens, vectors, and animal hosts is another factor influencing the epidemiology of zoonoses with a wildlife reservoir. Such movement can, for example, occur through human travel and trade, by natural movement of wild animals including migratory birds, and by anthropogenic movement of animals. For instance, infectious agents harbored within insects, animals, or humans can travel halfway around the globe in <24 hours in airplanes. Thus, infectious agents can be transported to the farthest land in less time than it takes most diseases to incubate. The appearance of West Nile virus infection in New York in 1999, and the subsequent spread within the United States, is an example of introduction and establishment of a pathogen that apparently originated in the Middle East (15).

Movement of infected wild and domestic animals is an important factor in the appearance of rabies in new locations. Rabies virus, which is widely distributed and affects various animals, especially canids, was introduced into North America by infected dogs in the early 18th century, with subsequent spillover to a variety of wild terrestrial mammals. Rabies became established in raccoons in the mid-Atlantic states in the late 1970s when raccoons were translocated from the southeastern United States, where rabies was endemic in this species (16). Finland experienced an outbreak of rabies linked to raccoon dogs in 1988. The raccoon dog had spread to Finland after this species was released in western Russia for fur trade. Rabies most probably arrived in Finland by wolves migrating from Russia during wintertime along the ice-packed coast (17). In the Arctic, the ice links the continents together. The movement of the arctic fox from the archipelago of Spitzbergen to Novaja Zemlja in Siberia and from Canada to Greenland has been described, indicating another way that rabies can be spread to new areas (18,19).

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Bovine tuberculosis caused by Mycobacterium bovis is another zoonosis in which both natural and anthropogenic movement of animals has influenced the epidemiology. This zoonosis is emerging in wildlife in many parts of the world, and wildlife can represent a source of infection for domestic animals and humans. Bovine tuberculosis was probably introduced into Africa with imported cattle during the colonial era and thereafter spread to and became endemic in wildlife (20). In Ireland and Great Britain, badgers maintain the infection, whereas the brushtail possum constitutes a main wildlife reservoir in New Zealand. In parts of Michigan, bovine tuberculosis is endemic among white-tailed deer, whereas in Europe, both wild boars and various deer species can be a reservoir of the pathogen. The natural movement of these reservoir animals increases the spread of the disease to domestic animals and thereby its public health impact (21) (reviewed by Kruse *et al.*, 2004).

The epidemiology of multilocular echinococcosis, caused by the small tapeworm Echinococcus multilocularis, has also been influenced by the translocation of animals. The main hosts are canids, especially foxes; the intermediate hosts are small rodents. Humans can become accidental intermediate hosts, by ingesting eggs. Multilocular echinococcosis occurs in large parts of the Northern Hemisphere. In 1999, E. multilocularis was detected for the first time in Norway, in the archipelago of Spitzbergen (10,22). The parasite most probably spread from Russia, by natural movement of the main host, the Arctic fox. Establishment of the parasite was possible because the intermediate host, the sibling vole, had previously been translocated to Spitzbergen, most likely through imported animal feed (23). In Copenhagen, Denmark, in 2000, E. multilocularis was detected in a traffic- killed red fox. The theory is that the fox had traveled by train from central

Europe, where the disease is endemic (H.C. Wegener, pers. comm.) (reviewed by Kruse *et al.*, 2004).

During the summer of 2003, an outbreak of monkeypox occurred in the United States with 37 confirmed human cases (24). Monkeypox is a rare zoonosis caused by a poxvirus that typically occurs in Africa. It was first found in monkeys in 1958 and later on in other animals, especially rodents. The African squirrel is probably the natural host. Transmission to humans occurs by contact with infected animals or body fluids. The cases in the United States, the first outside Africa, were associated with contact with infected prairie dogs. The outbreak was epidemiologically linked to imported African rodents from Ghana. Most likely, infected imported rodents have transmitted the virus to prairie dogs in United States. This transmission illustrates how non-native animal species can create serious public health problems when they introduce a disease to native animal and human populations. Thus, the transportation, sale, or distribution of animals, or the release of animals into the environment, can represent a risk for spread of zoonoses(reviewed by Kruse *et al.*, 2004).

Microbial changes or adaptation also influence the epidemiology of zoonoses with a wildlife reservoir. These changes include mutations, such as genetic drift in viruses; activation and silencing of genes; genetic recombinations, such as genetic shift in viruses; and conjugation, transformation, and transduction in bacteria. Natural selection and evolution also play a role. Transmission of adaptive or genetically changed microorganisms from wildlife to humans, either directly or indirectly through domestic animals, may occur in many ways. In this respect an international wildlife trade, often illegal, in which wild animals end up in live-animal markets, restaurants, and farms, is important because such practices increase the proximity between wildlife, domestic animals, and humans (25) (reviewed by Kruse *et al.*, 2004).

Severe acute respiratory syndrome (SARS) is a current example of likely microbial adaptation. This viral respiratory illness, caused by SARS-associated coronavirus, is believed to have emerged in Guangdong, China, in November 2002. SARS was first reported in Asia in February 2003, and over the next few months, the illness spread to a global epidemic before it was contained. According to the World Health Organization, 8,098 cases, including 774 fatalities, have occurred. The virus has an unknown reservoir, but wildlife is a likely source of infection. Natural infection has been demonstrated in palm civet cats in markets and also in raccoon dogs, rats, and other animals indigenous to the area where SARS likely originated (26) (reviewed by Kruse *et al.*, 2004).

Influenza A: Genetic changes typically influence the epidemiology of influenza A. Natural infections with influenza A viruses have been reported in a variety of animal species, including birds, humans, pigs, horses, and sea mammals, and its main reservoir seems to be wild waterfowl, especially ducks. Influenza A virus has two main surface antigens; hemagglutinin with 15 subtypes and neuraminidase with 9 subtypes. All these subtypes, in most combinations, have been isolated from birds, whereas few combinations have been found in mammals. In the 20th century, the sudden emergence of antigenically different strains transmissible in humans, termed antigenic shift, has occurred on four occasions, each time resulting in a pandemic. "New" pandemic strains most certainly emerged after reassortment of genes of viruses of avian and human origin in a permissive host (27). The H5N1 strain of a highly pathogenic avian influenza that caused a severe outbreak in poultry in Southeast Asia in 2004 (28) demonstrated its capacity to infect humans; 39 cases, 28 of them fatal, were officially reported (29). For the human population as a whole, the main danger appears to be simultaneous infection with an avian and a human influenza virus.

Reassortment could then occur either in humans or in pigs with the potential emergence of a virus fully capable of spread among

humans but with antigenic characteristics for which the human population was immunologically naïve (reviewed by Kruse *et al.*, 2004).

Enhanced recognition can also result in an apparent change in the epidemiology of a zoonosis, for example, the recognition of an agent that has been present for a long time but was previously undetected because of lack of diagnostic tools. Improved methods for molecular characterization have helped describe a larger repertoire of zoonotic agents (reviewed by Kruse *et al.*, 2004).

Recognition and emergence of human tickborne ehrlichiosis are recent and continuing events, beginning with human monocytic ehrlichiosis and human granulocytic ehrlichiosis, reported first in the United States in 1987 and 1994, respectively. The causative agents, Ehrlichia chaffeensis and Anaplasma phagocytophilum, are intracellular

bacteria that are maintained in zoonotic cycles involving persistently infected deer and rodents (30) (reviewed by Kruse *et al.*, 2004).

From 1994 to 2004, three zoonotic paramyxoviruses with a wildlife reservoir have emerged. The Hendra, Menangle, and Nipah viruses all have a fruit bat reservoir (31). Humans are infected by close contact with infected pigs or horses. Hendra virus infection was described in Australia in 1994, where it caused acute, fatal respiratory disease in horses and humans. Menangle virus was also described in Australia, in 1996, where it caused reproductive disorders in pigs and an influenza-like disease in humans. Nipah virus was detected in 1998, in Malaysia, when it caused severe disease with respiratory and neurologic symptoms among pigs and encephalitis with a 40% death rate in humans in close contact with pigs (reviewed by Kruse *et al.*, 2004).

Since 1994, when the isolation of Brucella spp. from marine mammals was reported for the first time, such infections have been detected in a wide range of marine mammal species and populations. The pathologic role of marine Brucella spp. in animals remains unclear, as does their zoonotic potential. In 2003, two human cases of community-acquired granulomatous central nervous system infections caused by marine Brucella spp. were reported (32) (reviewed by Kruse *et al.*, 2004).

Human behavior and demographic factors can also influence the epidemiology of zoonoses with a wildlife reservoir. Hiking, camping, and hunting are activities that may represent risk factors for acquiring certain zoonoses with a wildlife reservoir, e.g., tickborne zoonoses and tularemia. Eating habits can also play a role. For example, eating meat from exotic animals such as bear increases the risk of acquiring trichinellosis (33). AIDS represents a disease in which demographic factors and human behavior have contributed to its development into a global public health problem. The origin of HIV, the virus causing AIDS, is still a matter of controversy, but HIV likely spread to humans from nonhuman primates in West Africa (34) (reviewed by Kruse *et al.*, 2004).

Anthropogenic land use changes

Anthropogenic land use changes drive a range of infectious disease outbreaks and emergence events and modify the transmission of endemic infections. These drivers include agricultural encroachment, deforestation, road construction, dam building, irrigation, wetland modification, mining, the concentration or expansion of urban environments, coastal zone degradation, and other activities. These changes in turn cause a cascade of factors that exacerbate infectious disease

emergence, such as forest fragmentation, disease introduction, pollution, poverty, and human migration. Human-induced land use changes are the primary drivers of a range of infectious disease outbreaks and emergence events and also modifiers of the transmission of endemic infections (Patz *et al.* 2000). These land use changes include deforestation, road construction, agricultural encroachment, dam building, irrigation, coastal zone degradation, wetland modification, mining, the concentration or expansion of urban environments, and other activities. These changes in turn cause a cascade of factors that exacerbate infectious disease emergence, such as forest fragmentation, pathogen introduction, pollution, poverty, and human migration. These are important and complex issues that are understood only for a few diseases. For example, recent research has shown that forest fragmentation, urban sprawl, and biodiversity loss are linked to increased risk for Lyme disease in the northeastern United States (Schmidt and Ostfeld 2001). Expansion and changes in agricultural practices are intimately associated with the emergence of Nipah virus in Malaysia (Chua *et al.* 1999; Lam and Chua 2002), cryptosporidiosis in Europe and North America, and a range of food-borne illnesses globally (Rose *et al.* 2001). Road building is linked to the expansion of bushmeat consumption that may have played a key role in the early emergence of human immunodeficiency virus types 1 and 2 (Wolfe *et al.* 2000), and simian foamy virus has been found in bushmeat hunters (Wolfe *et al.* 2004) (reviewed by Patz *et al.*, 2004).

Land-Use Drivers of Infectious Disease Emergence

The emerging infectious diseases (EIDs) resulting from land use change can be entirely new to a specific location or host species. This may occur either from "spillover" or cross-species transmission or simply by extension of geographic range into new or changed habitats. More than 75% of human diseases are zoonotic and have a link to wildlife and domestic animals (Taylor *et al.*, 2001) (reviewed by Patz *et al.*, 2004).

The working group developed an extensive list of processes by which land use affects human health (specifically, infectious disease occurrence) and of other factors that contribute to this relationship: agricultural development, urbanization, deforestation, population movement, increasing population, introduction of novel species/pathogens, water and air pollution, biodiversity loss, habit fragmentation, road building, macro and micro climate changes, hydrological alteration, decline in public health infrastructure, animal-intensive systems, eutrophication, military conflict, mono-cropping, and erosion (ranked from highest to lowest public health impact by meeting participants). The four mechanisms that were felt to have the greatest impact on public health were changes to the physical environment; movement of populations, pathogens, and trade; agriculture; and urbanization. War and civil unrest were also mentioned as a potentially acute and cross-cutting driver. Infectious disease agents with the strongest documented or suspected links to land use change have been reviewed by Patz *et al.* (2004).

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Changes to the biophysical environment

Deforestation: Rates of deforestation have grown exponentially since the beginning of the 20th century. Driven by rapidly increasing human population numbers, large swaths of species-rich tropical and temperate forests, as well as prairies, grasslands, and wetlands, have been converted to species-poor agricultural and ranching areas. The global rate of tropical deforestation continues at staggering levels, with nearly 2-3% of forests lost globally each year. Parallel with this habitat destruction is an exponential growth in human-wildlife interaction and conflict. This has resulted in exposure to new pathogens for humans, livestock, and wildlife (Wolfe *et al.* 2000). Deforestation and the processes that lead to it have many consequences for ecosystems. Deforestation decreases the overall habitat available for wildlife species. It also modifies the structure of environments, for example, by fragmenting habitats into smaller patches separated by agricultural activities or human populations. Increased "edge effect" (from a patchwork of varied land uses) can further promote interaction among pathogens, vectors, and hosts. This edge effect has been well documented for Lyme disease (Glass *et al.* 1995). Similarly, increased activity in forest habitats (through behavior or occupation) appears to be a major risk factor for leishmaniasis (Weigle *et al.* 1993). Evidence is mounting that deforestation and ecosystem changes have implications for the distribution of many other microorganisms and the health of human, domestic animal, and wildlife populations (reviewed by Patz *et al.*, 2004).

One example of the effects of land use on human health is particularly noteworthy. Deforestation, with subsequent changes in land use and human settlement patterns, has coincided with an upsurge of malaria and/or its vectors in Africa (Coluzzi 1984, 1994; Coluzzi *et al.* 1979), in Asia (Bunnag *et al.* 1979), and in Latin America (Tadei *et al.* 1998). When tropical forests are cleared for human activities, they are typically converted into agricultural or grazing lands. This process is usually exacerbated by construction of roads, causing erosion and allowing previously inaccessible areas to become colonized by people (Kalliola and Flores Paitán 1998). Cleared lands and culverts that collect rainwater are in some areas far more suitable for larvae of malaria-transmitting anopheline mosquitoes than are intact forests (Charlwood and Alecrim, 1989; Jones, 1951; Marques, 1987) (reviewed by Patz *et al.*, 2004).

Another example of the effects of land use on human health involves deforestation and noninfectious disease: the contamination of rivers with mercury. Soil erosion after deforestation adds significant mercury loads, which are found naturally in rainforest soils, to rivers. This has led to fish in the Amazon becoming hazardous to eat (Fostier *et al.*, 2000; Veiga *et al.*, 1994) (reviewed by Patz *et al.*, 2004).

Habitat fragmentation: This alters the composition of host species in an environment and can change the fundamental ecology of microorganisms. Because of the nature of food webs within

ecosystems, organisms at higher trophic levels exist at a lower population density and are often quite sensitive to changes in food availability. The smaller patches left after fragmentation often do not have sufficient prey for top predators, resulting in local extinction of predator species and a subsequent increase in the density of their prey species. Logging and road building in Latin America have increased the incidence of cutaneous and visceral leishmaniasis (Desjeux, 2001), which in some areas has resulted from an increase in the number of fox reservoirs and sand fly vectors that have adapted to the peridomestic environment (Patz *et al.* 2000). Foxes, however, are not very important reservoirs for leishmaniasis in Latin America (Courtenay *et al.* 2002), and a more important factor in the transmission cycle includes domestic dogs (reviewed by Patz *et al.*, 2004).

Ostfeld and Keesing (2000) have demonstrated that smaller fragments in North American forests have fewer small mammal predators. Results suggest that the probability that a tick will become infected depends on not only the density of white-footed mice but also the density of mice relative to that of other hosts in the community. Under this scenario, the density effect of white-footed mice, which are efficient reservoirs for Lyme disease, can be "diluted" by an increasing density of alternative hosts, which are less efficient at transmitting Lyme disease. These results suggest that increasing host diversity (species richness) may decrease the risk of disease through a "dilution effect" (Schmidt and Ostfeld 2001) (reviewed by Patz *et al.*, 2004).

Extractive industries: Gold mining is an extractive industry that damages local and regional environments and has adverse human health effects, because mercury is used to extract gold from riverbeds in the tropical forests. Not only does mercury accumulate in local fish populations, making them toxic to eat (Lebel *et al.* 1996, 1998), but mercury also suppresses the human immune system. Also, in gold-mining areas, more mosquito-breeding sites and increased malaria risk result from digging gem pits in the forest and from craters resulting from logging; broader disease spread occurs as populations disperse throughout the region (Silbergeld *et al.* 2002) (reviewed by Patz *et al.*, 2004).

Movement of populations, pathogens, and trade

The movement of humans, domestic animals, wildlife populations, and agricultural products through travel, trade, and translocations is a driver of infectious disease emergence globally. These sometimes inadvertent, sometimes deliberate movements of infectious disease and vectors (e.g., the introduction of smallpox and measles to the Americas by Spanish conquistadors) will continue to rise via continually expanding global travel and by development of Third World populations. Human introduction of pathogens, hosts, or materials into new areas has been termed "pathogen pollution" (Daszak *et al.* 2000) (reviewed by Patz *et al.*, 2004).

Land use changes drive some of these introductions and migrations and also increase the vulnerability of habitats and populations to these introductions. Human migrations also drive land use changes that in turn drive infectious disease emergence. For example, in China's Yunnan Province, an increase in livestock populations and migration has led to an increase in the incidence of schistosomiasis (Jiang *et al.* 1997). In Malaysia, a combination of deforestation, drought, and wildfires has led to alterations in the population movements and densities of flying foxes, large fruit bats known to be the reservoir for the newly emergent zoonosis Nipah virus (Chua *et al.* 1999). It is believed that the increased opportunity for contact between infected bats and pigs produced the outbreak of the disease in pigs, which then was transmitted to people in contact with infected pigs

(Aziz *et al.* 2002) (reviewed by Patz *et al.*, 2004).

Another example of human-induced animal movement on a much larger scale is the international pet trade. This movement of animals involves many countries and allows for the introduction of novel pathogens, such as monkeypox, with the potential to damage ecosystems and threaten human and animal health. Monkeypox was originally associated with bushmeat hunting of red colobus monkeys (Procolobus badius); after a localized epidemic emerged in humans, monkeypox persisted for four generations via human-to-human contact (Jezek *et al.* 1986) (reviewed by Patz *et al.*, 2004).

Human movement also has significant implications for public health. Not only are travelers (tourists, businesspeople, and other workers) at risk of contracting communicable diseases when visiting tropical countries, but they also can act as vectors for delivering infectious diseases to another region or, in the case of severe acute respiratory syndrome (SARS), potentially around the world. Refugees account for a significant number of human migrants, carrying diseases such as hepatitis B and tuberculosis and various parasites (Loutan *et al.* 1997). Because of their status, refugees become impoverished and are more exposed to a wide range of health risks. This is caused by the disruption of basic health services, inadequate food and medical care, and lack of clean water and sanitation (Toole and Waldman 1997). People who cross international boundaries, such as travelers, immigrants, and refugees, may be at increased risk of contracting infectious diseases, especially those who have no immunity because the disease agents are uncommon in their native countries. Immigrants may come from nations where diseases such as tuberculosis and malaria are endemic, and refugees may come from situations where crowding and malnutrition create ideal conditions for the spread of diseases such as cholera, shigellosis, malaria, and measles [Centers for Disease Control and Prevention (CDC) 1998] (reviewed by Patz *et al.*, 2004).

Surveillance

Therefore, development of appropriate programs for surveillance and for monitoring emerging diseases in their wildlife reservoirs is essential. Most animal pathogens for which surveillance programs exist relate to farm animals, and few or no programs are specifically aimed at wildlife. Two different but complementary approaches are 1) to monitor the presence of specifically identified pathogens that have emerged as human pathogens and 2) to investigate in a given wildlife species the presence of known or unknown infectious agents. Furthermore, conservation of habitat biodiversity is critical for preventing emergence of new reservoirs or amplifier species. Key measures for reducing the dispersion of emerging zoonoses include sustainable agricultural development, proper education of tourists about the risks of outdoor activities, and better control of the live animal trade (exotic pets, wet markets, bushmeat). Public health services and clinical practitioners (physicians, veterinarians) need to more actively educate the public about the risks of owning exotic pets and adopting wild animals.

As suggested by Kuiken *et al.* (2005), it is time to form "a joint expert working group to design and implement a global animal surveillance system for zoonotic pathogens that gives early warning of pathogen emergence, is closely integrated to public health surveillance and provides opportunities to control such pathogens before they can affect human health, food supply, economics or biodiversity." Major tasks that should be taken by the international community include better integration and coordination of national surveillance systems in industrialized and developing coun-

tries; improved reporting systems and international sharing of information; active surveillance at the interface of rural populations and wildlife habitats, especially where poverty and low income increase risks for pathogen transmission; training of professionals, such as veterinarians and biologists, in wildlife health management; and establishment of collaborative multidisciplinary teams ready to intervene when outbreaks occur (Chomel *et al.*, 2007).

Health Evaluation of Free-ranging and semi-captive Orangutans (Pongo Pygmaeus Pygmaeus) in Sabah, Malaysia

Baseline data on health of free-ranging wildlife is essential to evaluate impacts of habitat transformation and wildlife translocation, rehabilitation, and reintroduction programs. Health information on many species, especially great apes, is extremely limited. Between 1996 and 1998, 84 free-ranging orangutans captured for translocation, underwent a complete health evaluation. Analogous data were gathered from 60 semi-captive orangutans in Malaysia. Baseline hematology and serology; vitamin, mineral and pesticide levels; and results of health evaluations, including physical examination, provide a baseline for future monitoring. Free-ranging and semi-captive orangutans shared exposure to 11 of 47 viruses. The semi-captive orangutans had significantly higher prevalence of antibodies to adenovirus ($P<0.0005$) and rota (SA 11) virus ($P<0.008$). More free-ranging than semi-captive animals had antibodies to Japanese encephalitis virus ($P<0.08$) and foamy virus ($P=0.05$). Exposure to parainfluenza and langat viruses was detected exclusively in semi-captive animals and exposure to sinbis virus was only found in free-ranging orangutans. There was evidence of exposure to respiratory syncytial virus, coxsackie virus, dengue virus, and zika virus in both groups. Ebstein-Barr virus was ubiquitous in both groups. Prevalence of antibodies against mumps virus changed from 0% in 1996 to 45% in 1998. No antibodies were detected to many important zoonotic viral pathogens, including herpesvirus and hepatitis virus. Prevalence of Balantidium coli and Plasmodium pitheci infections and exposure to mycobacterium was higher in the semi-captive animals. Differences in exposure to pathogens between the groups may be due to environmental factors including differences in exposures to other species, habitat quality, nutritional status, and other potential stressors. Differences in health parameters between captive and free-ranging orangutans need to be considered when planning conservation areas, translocation procedures, and rehabilitation protocols. Because survival of the orangutan is linked to animal and ecosystem health, results of this study will assist wildlife conservation programs by providing baseline health information (Kilbourn *et al.*, 2003). Such similar studies are required for ? if not for all ? at least important zoonotic agents which threaten the wildlife most.

Wildlife Zoonoses for the Veterinary Practitioner There is increasing concern regarding wildlife zoonoses. It is important that veterinarians are able to give their clients and the general public appropriate advice regarding the potential risks, what to do in the event of possible exposure, input regarding early recognition of the signs of wildlife zoonotic infections, and practical advice to prevent or reduce exposure to wildlife zoonotic pathogens. This advice is summarized for a variety of wildlife zoonoses in North America including rabies, hantavirus, tularemia, plague, psittacosis, baylis ascaris, alveolar echinococcosis, arthropod-borne encephalitis, tick-borne diseases, and food safety related to game meat (Sleeman, 2006).

Prevention and Control

Although prevention and control strategies for the various zoonoses associated with wildlife share many common aspects, specific strategies are also needed to address the etiology and epidemi-

ology of the disease, characteristics of the pathogen involved, ecologic factors, and the population at risk. As wildlife is an essential component in the epidemiology of many, if not most, zoonoses, wildlife should be taken into account in the risk analysis framework. Consequently, cost-effective prevention and control of zoonoses in humans, including risk communication, necessitate an interdisciplinary and holistic approach that acknowledges the importance of wildlife as a reservoir (reviewed by Kruse *et al.*, 2004).

To increase the capability of recognizing zoonoses with a wildlife reservoir, better national surveillance systems for humans and animals are needed, as well as better international integration and sharing of information from such systems. Which diseases should be reportable also needs to be evaluated on a continuous basis. Improved reporting

systems and screening programs for human infections, including the application of syndromic surveillance, are warranted to detect new and emerging zoonoses. Efficient surveillance is dependent upon a laboratory system that is capable of identifying and characterizing the pathogens in question. More research is needed to better understand the epidemiology and pathogenesis of various zoonoses, to improve diagnostic methods, and to develop cost-effective vaccines and drugs. Training and education are prerequisites to enable the personnel involved at the various stages, from field to laboratory personnel, to detect zoonoses, both new and old (reviewed by Kruse *et al.*, 2004).

Information and communication are key components in any prevention and control strategy. Public education and behavioral change are also important factors for successful

intervention. Implementing restrictions on anthropogenic animal movement is another important preventive measure (reviewed by Kruse *et al.*, 2004).

For vector-borne zoonoses, vector control should be an integral part of any intervention strategy (reviewed by Kruse *et al.*, 2004).

Interdisciplinary and international collaboration is necessary for the rapid identification and effective management of outbreaks of zoonoses. The pivotal role of international organizations such as World Health Organization and Office International des Epizooties is becoming clearer, exemplified by the 2004 avian influenza outbreak in Southeast Asia. Containing zoonoses with a wildlife reservoir relies on efficient national, regional, and international cross-sectional networks that can improve data sharing and thereby alertness and the timely and effective response to disease outbreaks (reviewed by Kruse *et al.*, 2004).

Future challenges and thrusts

The recommendations stemming from the international colloquium are highly relevant to the Millennium Ecosystem Assessment (MEA), a broad multiagency/foundation-sponsored scientific assessment of degraded ecosystem effects on human well-being. A conceptual framework of the MEA already provides an approach to optimize the contribution of ecosystems to human health (MEA 2003). The recommendations emerged (Patz *et al.*, 2004) are described below:

- Conceptual model: bringing land use into public health policy
- Research on deforestation and infectious disease
- Policies to reduce microbial traffic/pathogen pollution

- Centers of Excellence in Ecology and Health Research and Training
- Implementing research and policy programs
- Addressing trade-offs among environment, health, and development

As the working group of researchers continues to work on these topics, we face three challenges. First, strong trans-disciplinary research partnerships need to be forged to approach the research with the degree of creative thinking and comprehensiveness required by the nature of the problems. Second, if the work is to influence policy, the choice of questions and the research must be undertaken collaboratively with the local community and also through discussion with decision makers in government, industry, civil society, and other sectors. Third, investigators must consider how they can integrate their findings into the social, economic, and political dialogue on both the environment and health, globally and locally. As links between land use and health are elucidated, an informed public will more readily use such discoveries to better generate political will for effective change (Patz *et al.* 2004).

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SESSION V

Role of Genomics & Proteomics in development of Biologicals

Chairman : **Dr. G. Buthchaiah**

Co-Chairman : **Dr. H.M. Saxena**

Rapporteur : **Dr. Rashmi Singh**

LEAD PAPERS

V.1 Self Replicating Gene Vaccines: Development and Scope in Animal Viral Disease Control Using FMD as a Model System.
Sarika, S., Nagarajan, C., Dechamma, H.J., Reddy, G.R and Suryanarayana, VVS.

V.2 Infectious Diseases and Wildlife Biodiversity Conservation
Dharmeswar Das

V.3 Metagenomics: A Genomic Tool for Microbial Diversity
M.L. Sangwan, Tanuj Ambwani, R.A. Siddique and Anand Kumar

ABSTRACTS

5.01 Studies on antihypertensive & immunomodulatory bioactive peptides derived from goat casein
Santosh Kumar, H.K.L. Tandon, Suman Kapila

5.02 SDS PAGE and Western blotting to compare between mutant and non mutants of *Salmonella* Abortusequi
Mudit Chandra, B. R. Singh, S. K. Srivastava, P. Chaudhary, M. Z. Siddiqui, Ravi Kant Agarwal, Amit Verma

5.03 Molecular confirmation of virulence of *Bacillus cereus*
Rajguru A.S., Karpe A.G., Niture G.S. & Modekar S.S.

5.04 Pineal-Adrenal-Immune system relationships under thermal-stress: Effect of pineal proteins on phagocytic activity in chemically adrenalectomized goats
V. Sejian, R.S. Srivastava, & V.P. Varshney

5.05 Molecular characterization of field isolates of Inclusion Body Hepatitis virus
Subodh Kumar Saxena, Vipan, Rajesh Kumar and Rajesh Chandra

5.06 Cloning of Hexon gene of a field isolate of Inclusion Body Hepatitis Virus
Dilip Kumar, Vipan, Rajesh Kumar, Nishant S. Saini and Rajesh Chandra

5.07 Identification of animal species using PCR-based molecular techniques
Nagappa K, S.P. Singh and Umapathi V.

5.08 SDS-PAGE profile of OMPs from *E. coli* grown under planktonic and biofilm mode
Vivek Prabhu, S. Isloor, D. Rathnamma, Kavita G. & Sadish

5.09 Antibiotic sensitivity and plasmid profiling of avian *Escherichia coli*
T.V. Shiva Shankar, Archana Sharma, Y.B. Rajeshwari

5.10 Diagnosis of benzimidazole resistance in *Haemonchus contortus* using allele-specific PCR technique and its use in genotyping of worms
Rajat Garg & C.L. Yadav

5.11 Comparative studies on apoptosis induced by field and vaccine strain of IBDV in poultry
M. Pandey, D.K. Agrawal, V. Umapathi,

5.12 Recent Trends in High-Throughput Screening in Drug Development
S.P. Singh & Tanuj Ambwani

5.13 Screening of Enterotoxin gene in *Salmonella* serovars isolated from Gangetic water
Gunjan Srivastava, M.K. Saxena

5.14 Isolation, plasmid profiling and MDR analysis of rare isolates of Salmonella isolated from Ganga water
Kalaiyarasu.S, R.S.Gupta & Mumtesh Kumar

5.15 Comparison of protein-profile of *Echinococcus granulosus*, *Taenia hydatigena* and *Dipylidium caninum* of dogs by SDS-PAGE
Ananda, K.J., Javare Gowda., Placid E.D'Souza, V.V.S. Suryanarana & Shrikrishna Isloor

5.16 Role of complement in killing of *Pasturella multocida* B: 2 strains
Deepti Chachra, John Coote, Roger Parton, S.K.Jand and A.K.Arora

5.17 Studies on different virulence factors of Avian Pathogenic *Escherichia coli* (APEC)
T.V.Shiva Shankar[1], Archana Sharma[1], Y.B.Rajeshwari[2]

5.18 Multiple Antibiotic Resistance Analysis in Poultry *Salmonella* Isolates
R. A. Siddique, Mumtesh Saxena

5.19 RAPD Analysis in Poultry *Salmonella* Isolates
R. A. Siddique, Mumtesh Saxena,Tanuj Ambwani and B.D. Lakhchaura

5.20 Lytic Bacteriophage therapy to control pathogenic *Staphylococcus aureus*
Ragini Gupta, Mayank Rawat, Suresh Kannan

5.21 Apoptosis induction in vero cells by Newcastle disease virus requires virus entry replication and *de-novo* protein synthesis
P.V. Ravindra, Ashok K Tiwari, Barkha Ratta, Uttara Chaturvedi, Sudesh Kumar Palia, Prasant Kumar Subudhi, Rajiv Kumar, Bhaskar Sharma, Anant Rai and RS Chauhan

5.22 HN protein of Newcastle Disease Virus causes apoptosis in Chicken Embryo Fibroblast cells
Ravindra PV; Ashok K. Tiwari, Bhaskar Sharma, Yogendra Singh Rajawat, Barkha Ratta, Sudesh palia, Sundaresan N, Uttar Chaturvedi, Aruna Kumar GB, Kantaraja Chindera, Meeta saxena, PK Subudhi, Anant Rai, Chauhan RS

SELF-REPLICATING GENE VACCINES: DEVELOPMENT AND SCOPE IN ANIMAL VIRAL DISEASE CONTROL USING FMD AS A MODEL SYSTEM.

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In recent years, naked DNA vaccination has emerged as one of the most promising venues for recombinant vaccine. The concept is based on inoculation of plasmids encoding a gene or genes under a strong eukaryotic promoter, e.g. the early CMV promoter/enhancer or Alpha virus (Atkins *et al.*, 1999). Following vaccination and DNA entry into the cell, expression of the gene occurs. This permits the entry of the newly expressed and processed protein into the class 1 major histocompatibility complex (MHC) antigen presentation pathway through the post-translational process of cleaving the endogenous protein into 9 – 11 amino acid long peptides. Presentation of the processed proteins resulted in eliciting CD8+ cytotoxic T lymphocyte (CTL) response (McClements *et al.*, 1996). The induction and involvement of antibodies upon DNA vaccination has also been demonstrated in mice, bovine and nonhuman primates (Koletzki *et al.*, 2000, Vordermeier *et al.*, 2000, and Couillin *et al.*, 2001). Thus, the immune response to DNA vaccines seems to be analogous to that induced by conventional live attenuated virus (LAV) vaccination. In both cases, viral antigens are expressed and processed in the cells (LAV through normal viral replication, and DNA vaccines as a result of expressing the recombinant antigen) and presented to the immune system in such a way that it induces both humoral as well as cell-mediated immunity.

While several attempts have been made to develop potent gene vaccine against several diseases including FMD, the encouraging results could be obtained only in a few cases indicating the strategies have much to be improved upon. Right from the early reports of Ward *et al.*, (1997) and Chinsangaram *et al.*,(1998) , to Wong *et al.*, (2002), the approaches to develop DNA vaccines against FMDV have seen through several modifications seeking to improve upon the immune responses thus elicited. One of these is to achieve the replication of the RNA transcript of the antigen gene in the injected cells by co-expressing RNA dependent RNA polymerase, which can be achieved through Alpha virus based vectors.

The unique characteristics of Alpha viruses make them attractive candidates for developing expression systems used in DNA/RNA based vaccines. For example, size of positive sense SS Alpha viral genome (encoding its own replicase) is 11.7 Kb, which facilitates the construction of recombinant cDNA clones. The, productive replication in this case can be initiated either by infection of the cell or by transfection of the genomic RNA into the cytoplasm of the cell. In both cases, vigorous replication leads to high-level production of new virus particles (as many as 10^5 per cell). Theoretically up to 2,00,000 copies of RNA can be produced in a single cell with in 4 hr and the expression of the encoded antigen can be as much as 25% of total cell protein (Rolls *et al.*, 1994). The structural proteins of the virus are expressed from a sub genomic RNA which is collinear with the last third of the genomic sequences. This has allowed the manipulation of the sub genomic sequences without affecting the replication capacity of the system. Additionally, expression is under the control of the virus (not the host), and the lack of a DNA phase precludes the possibility of cell transformation. They do not appear to down modulate the immune system to the same extent as several large DNA viruses (Ploegh, 1998). Consequently, there is a great deal of interest in the use of RNA viruses to induce protective immune responses, which could include the reinforcement of mucosal defense. Foreign sequences can be placed in front or after the structural

genes of the virus in the context of an otherwise wild type genome. Such constructs tend to be unstable upon passaging and have the additional drawback that large amounts of viral structural proteins are produced. Therefore, the most preferable strategy is to replace the virus structural genes with the gene of interest. Due to the efficient production of the foreign mRNA by the viral transcriptase, large amounts of foreign protein are produced. Such alpha viral expression system is now in the use of a DNA-based strategy where the re-alpha virus replicon sequences have been put under a eukaryotic promoter such as the CMV immediate early promoter (pCMV). In this system, DNA is directly transfected into the cell where the recombinant construct is transcribed by the host RNA polymerase. The RNA is transported into the cytoplasm where viral replicase is produced and then takes over the replication of the RNA into new copies and also transcribes the foreign sequences.

The benefits of this strategy are low costs (no *in vitro* transcription required), a more stable molecule, and no risk of producing replication-proficient virus. Preliminary results from cell culture experiments have shown that the DNA system gives at least 10 fold higher expression levels as compared to conventional naked DNA delivery methods (using pCMV plasmids) (Herweijer *et al.*, 1995; Dubensky *et al.*, 1996). Alpha virus vectors, which are derived from self-replicating RNA viruses when used as vaccines, have been shown to be significantly more effective in inducing protective immunity than conventional genetic vaccines (Xiong *et al.*, 1989).

Immune Responses To Self Replicating Genetic Vaccines:

A major rationale for putting antigen –coding genes under the control of alpha viral RNA replicase was to enhance antigen expression and presentation. A fundamental difference between replicase-based DNA vaccines and conventional DNA vaccines is the occurrence of virus-like RNA replication inside transfected host. Transfection of host cells with replicase-based genetic vaccines could trigger a series of danger signals (Matzinger, 1998). Replicase-based DNA or RNA induces apoptotic death of the host cell *in vitro* just as alpha viral infection induces apoptosis in host cells (Ying *et al.*, 1999). These apoptotic cells may be picked up by dendritic cells to present to the immune system (Albert *et al.* 1998). Transfection with self-replicating genetic vaccines may also cause the production of heat shock proteins, in transfected or by-stander cells (Chelbi-Alix and Sripati 1994). The activity of the viral replicase may provide a powerful adjuvant effect because of the production of double stranded RNA intermediates.

Double stranded RNA itself is a potent inducer of the interferon and virus derived ds RNA can function as a strong adjuvant for cellular and humoral immune responses (Wright and Alder more 1985). Several molecules are known to bind to and can be activated by dsRNA. The best characterized are 2'-5' oligo adenylate (2-5A) synthetase and protein Kinase –RNA activated (PKR). The 2-5A system contributes to the antiviral effect of the interferons through the synthesis of 2-5A and its activation of RNAse, which degrades both viral and cellular RNA.PKR-expression, both induces and is induced by the interferons. PKR is then activated by dsRNA to phosphorylate its substrates, including e-IF2. This results in the inhibition of translation, further diminishing viral replication. The cellular death observed in response to ds RNA, is likely to be mediated by both the 2-5A system – induced RNAse as well as substrates of PKR (Rivas *et al.* 1998). Interferon – gamma potentiates the apoptotic effects of dsRNA (Tanaka *et al* 1998). Thus these vectors have high potentiality for being considered as media for the development of new generation vaccines for those diseases which hitherto were mostly unsuccessful due to several virus related factors like

poor immunogenicity, thermal stability and requirement of multiple immunological responses.

Foot and mouth disease (FMD) is one of such diseases, which need a promising vaccine, whose preparation does not involve the production of live virus. It is one of the most contagious and economically important diseases of cloven-footed animals such as cattle, buffalo, sheep and goats. The disease causes high morbidity and low mortality in the infected animals resulting in reduction in milk yield, draught power, repeat breeding and abortions. The disease is a major threat to dairy and livestock industry.

The disease can be controlled by regular vaccination using suitable vaccines, which is cost effective, safe and provides long lasting immunity. Conventional vaccine produced from the chemically inactivated virus, although potent in eliciting immune response, can meet only a few requirements of an ideal vaccine. At the initial stage of recombinant DNA technology it was hoped that the immunogen produced in heterologous host system could be used as safe and potent vaccines against FMD like several other afflictions. However the initial hype has come down when it was realized that the quantity and quality of host immune response are more important for protection and all components of immune system need to be activated. However it is understood beyond doubt that it is possible to achieve such response if the vaccine candidate is an attenuated organism and can replicate in the host system with no pathogenicity. Keeping this in view and taking subsequent advances in molecular immunology and recombinant DNA technology, several laboratories including IVRI, Bangalore Campus have initiated work on the development of newer vaccines for FMD. These include the live viral vectored vaccines (Srinivas 2005), self-replicating gene vaccines (Nagarajan 2004), Gamma Interferon gene adjuvanted vaccines (Ravikumar 2004 and DNA vaccine comprising genome length cDNA (Saravanan 2004, Ashok kumar, 2007)

The immunogen administered in soluble form, such as recombinant protein, generally induces only antibody responses. However DNA vaccines efficiently engage both MHC-I and MHC-II pathways allowing for the induction of CD8+ and CD4+ T cells. However, DNA vaccination in many cases is hampered by poor stability of injected DNA and thereby low efficiency in gene expression Therefore it is essential to improve the efficiency of DNA vaccines by including suitable delivery systems, gene adjuvants and/or making them self replicating in the host system. The work presented here deals with the development of Self Replicating DNA vaccines for FMD.

This Self Replicating vaccine candidates like viral vectors take the advantage of the replication machinery used by members if the Alpha virus genus, which includes Sindbis Virus, Semiliki Forest virus (SFV), and Venezuelan equine encephalitis (VEE) virus.

In our Lab two different self-replicating vaccines were developed, one for eliciting cell mediated immunity and the other for eliciting humoral immune response. In both the constructs the replicase gene from Sindbis vector was used. In order to drive the expression of the replicase gene two separate constructs were made, one carrying Cytomegalovirus promoter (CMV) (Nagarajan 2004) and the other carrying eukaryotic elongation Factor (eEFI) promoter. (Suryanarayana et al 2006)

The CMV promoter along with the T7 promoter sequences were retrieved from pcDNA vector and cloned upstream of the replicase gene in the Sin His vaccine vector. Poly (A) signal sequences of Bovine Growth Hormone was introduced downstream of the replicase gene. The vaccine vector pSin CMV Vac was used for the insertion of FMDV immunoreactive protein genes.

Since three major serotypes of FMDV (A, O and Asia1) are in circulation in India we have linked the immunoreactive protein genes of all the serotypes for constructing polyvalent vaccine construct (Nagarajan 2007). The linked polyprotein gene was expressed and the stability of the protein was confirmed by expression studies. The construct was subjected to transfection in BHK21 cell monolayer and the expression of the antigenic protein was confirmed by SDS-Page and Western Blotting.

It has been reported that humoral response is also important for protection against FMD. So we constructed another Self Replicating Vaccine vector for humoral response using pVac (Invivo gen, USA) vector as backbone. pVac is a DNA vaccine vector carrying of IL2 signal sequence of 21 amino acids for the secretion of antigenic proteins. The vector also carries a C-terminal transmembrane anchoring domain of the placental alkaline phosphates, which helps in anchorage of the secreted protein. A multiple cloning site is located downstream of the eEFI promoter for convenient cloning of the antigenic gene. Antigenic proteins are targeted and anchored to the cell surface by cloning the gene of interest in frame between the IL-2 signal sequences and anchoring signal sequences. The expressed protein is secreted and anchored to the cell membrane since MCS is between the secretory signal and the anchoring signal sequences.

Self-Replicating pVac vector was constructed by insertion of Replicase gene of Sindbis virus along with the sub-genomic promoter in such a way that the replicase gene product remains in the cytoplasm. The poly protein gene construct of FMD was inserted downstream of the Replicase gene in the vaccine vector to get pVacSelf Rep 990. Expression of the pVac Self Rep 990 cassette driven by the eEF1 promoter was studied by transfecting the clone into BHK21 cell monolayer. Comparative study of the Self Rep construct with non-self replicating clone (pVac 990) was also carried out. SDS-Page and Western Blotting analyzed the transfected samples. The expression of the FMD specific antigen in BHK cells was studied using S^{35}-methionine and confirmed that the level of expression in case of Self Rep vaccine vector was higher than non-self replicating construct.

Dose response and efficacy of the vaccines were tested in guinea pigs. Humoral response was studies by ELISA and serum neutralization studies. The quantity of the DNA construct needed for better humoral response was found to be 1 ug per dose. Self replicating gene Vaccines have additional advantages like low quantity of DNA requirement for achieving high immune response, no risk of DNA integration and expression of gamma interferon due to the dsRNA formation, which may be required for eliminating carrier status. The strategy used for construction of these vaccines and their immune response in experimental animals will be discussed.

Acknowledgement:

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INFECTIOUS DISEASES AND WILDLIFE BIODIVERSITY CONSERVATION

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Biodiversity is the product of the biological evolution. Geneticists are unanimous in their view that the biological evolution works through mutation, migration, selection and random genetic drift-all of which contribute to genetic diversity of life. The idea is to induce more and more variation so that life can adopt well in varied ecosystems and the changing environment over the time and space. Biodiversity, a concept used to describe the range of living organisms in a given area, takes into consideration the variety of life forms, the genes they contain, and the ecosystems they form. Life forms within an ecosystem vary in their size and shape from the simplest unicellular prokaryote, the bacteria, to the more complex multicellular eukaryotic organisms, such as elephants or bottlenose dolphins. Each organism plays an important role and contributes to ecosystem stability.

Wild life biodiversity - our Natural heritage:

Wildlife biodiversity is a natural heritage of mankind. This natural heritage if lost cannot be regained. The planet earth is having a spectrum of ecological social, cultural and biological diversity. In the past, historically living in harmony with nature, mankind directly or indirectly, contributed to the conservation of biological diversity. However, this ethos of conservation seems to have lost their significance in the present era of science and technology leading to a situation in which loss of species and biotypes has assumed alarming rate. Lately it has been realized all over the world that biodiversity should be regarded as a vital resource on which the present and the future well being of humanity will depend.

Many of the most highly vulnerable endangered species are found in developing countries, which do not have the required resource and expertise to establish their own gene banks either in-situ or ex-situ of desired level. It would seem a good idea to conserve the genetic material/ germ plasm of selected wild animals as near to the country of origin as possible involving the national conservation authorities in its collection and breeding management. Unfortunately very few National Parks and other designated protected areas are big enough to support adequate populations of important species without the risk of a slow erosion of genetic variability. One way of correcting this situation is by the regular introduction of fresh germ plasm, either in the form of live individual animals from unrelated distant sources (where possible) or indirectly using cryopreserved material. Zoos having the space and funds to keep the minimum required 50 "effective" number of breeding animals of one species can ensure presence of desired genetic variance for conservation of a species. The possibility of establishing wild animal gene bank on a global scale can also be explored.

Causes of loss of wildlife biodiversity:

Some of the major causes of declining wild life-population are Loss and fragmentation of habitats, poaching, poisoning, wildlife diseases (interrelated with habitat destruction), biological hazards (pollution, industrial waste) etc. The major endangered species of wild life in India are Royal Bengal tiger, Great Indian Rhinoceros, Indian elephant, Leopard, Asiatic lion, Barasingha, Gour (Indian bison), Sarus crane, Ganges river dolphin, Nilgiri tahr, King cobra, Red panda, Hoolock gibbon, Slow loris, Golden langur, Assamese macaque, Pink head duck, White winged wood

duck, Pigmy hog, Snow leopard etc. Various Legislations and Forest Acts for conservation of wildlife have been introduced from time to time.

With the advancement of science and technology man has started exploiting the natural resources far too in excess than its requirement affecting the intricate balance of the planetary ecosystem threatening the biosphere. As the Supreme Being, it is the mandatory duty of man to see that the biodiversity is preserved and life continue to live.

Conservation approaches:

Some measures of wild animal conservations are as follows:

In situ Conservation: This is the method in which the animals are maintained in their own habitats and in parks with habitat management.

Ex situ conservation: Captive breeding, gene and seed banks, zoos and aquaria and all other forms of maintaining species artificially and off-site.

Introductions: Releasing animals (captive or wild born) where they never existed usually because old habitat is lost or degraded, not available, but the new habitat is considered suitable.

Reintroductions: Releasing captive born animals where they once existed. This becomes successful only when the cause(s) of the original population decline are corrected.

Translocations: Moving wild-born animals from one place to another. This is done when the wild population is in imminent danger of extinction due to habitat alteration.

Captive breeding: Captive breeding and subsequent re-introduction of a threatened species is an important and in some cases very successful tool for species conservation.

The captive breeding programmes fit nicely into their dual objectives of public education and the preservation of species diversity. The use of captive breeding in species recovery has grown enormously in recent years, but without a concurrent growth in appreciation of its limitations. Captive breeding can play a crucial role in recovery of some species for which effective alternatives are unavailable in the short term. However, it should not displace habitat and ecosystem protection nor should it be invoked in the absence of comprehensive efforts to maintain or restore populations in wild habitats. Zoological institutions with captive breeding programmes should operate under carefully defined conditions of disease prevention and genetic/behavioral management. More important, these institutions should help preserve biodiversity through their capacities for public education, professional training, research, and support of in situ conservation efforts.

There are many success stories of captive breeding in conservation efforts of wild life. Captive breeding has saved the bison. Wolves roam Yellowstone and the Upper Peninsula of Michigan, the Peregrine Falcon is off the endangered species list, golden-lion tamarins thrive in the Brazilian forests, whooping cranes perform their mating dances along river banks in the west, and many more species might similarly be rescued. Extinct as wild populations, the European bison and Przewalski's horse owe their survival to private herds maintained by zoos and wealthy Europeans. The remaining wild California condors and black-footed ferrets were captured in a desperate and successful attempt to breed captive animals for release into the wild. Zoos, botanical gardens and aquaria have found new purpose and direction, providing a safety net when other protective measures have failed.

Animal reintroductions and translocations are potentially important interventions to save species from extinction, but most are unsuccessful. Mortality due to predation is a principal cause of failure. Animals that have been isolated from predators, either throughout their lifetime or over evolutionary time, may no longer express appropriate antipredator behavior.

Prevention of critical habitat loss is the wildlife biologist's preferred management method. There is a tendency for the public to believe that animals can be warehoused in zoological parks until habitat is restored and the wild populations reintroduced. Some of the conservation projects for endangered wildlife species initiated and implemented in India at different locations are for Indian one horned rhinoceros -Kaziranga National Park, Pigmy hog- Basistha, Guwahati, Kasmir Stag - Dachigam Sanctuary, Snow leopard – Ladakh, Sangai - Lamjao N.P., Swamp deer - Dudwa N.P., Wild Ass - Runn of Kutch, Lion tailed macaque - Western Ghats.

Problems encountered in Conservation efforts:

There are many problems and constraints encountered in conservation of wild life biodiversity. Some of the significant problems are –

(1) Establishing self-sufficient captive populations,

(2) Poor success in reintroductions,

(3) High costs,

(4) Domestication,

(5) Preemption of other recovery techniques,

(6) Disease outbreaks, and

(7) Maintaining administrative continuity.

Diseases and wildlife conservation:

Disease outbreaks both in wild and captive wild animal population are one of the major factors affecting their survival rate. Some of the common bacterial diseases encountered in wild animals are (i) Anthrax, (ii) Salmonellosis, (iii) Tuberculosis, (iv) Pasteurellosis, (v) Clostridial infection, (vi) Brucellosis; Viral diseases are (i) Rabies, (ii) FMD, (iii) Elephant pox, (iv) Rinderpest, (v) Blue tongue, (vi) Feline panleukopenia, (vii) Kayasanur forest disease. The parasitic diseases are (i) Helminthiasis (Strongylosis, Fasciolosis and Filariasis), (ii) Cestodes, (iii) Ectoparasites, (iv) Protozoal infections (Trypanosomiasis, Surrah). Some of the non-specific diseases are (i) Foot diseases (Kurry in elephants), (ii) Injury, (iii) Abscesses.

Table: Some major epizootics in India.

Year	Diseases	Animals affected
1871	Rinderpest	Deer, Wild buffaloes, Gaurs, Wild boars, Black buck, Barasingha
1935	FMD	Entire herd of Gaur wiped out in the Hyderabad State
1949	Anthrax	150 Wild elephants in Assam
1957	Anthrax	Killed many Gaurs, Deer & Wild boars in Goalpara district of Assam
1960	African horse sickness	Caused heavy mortality among the Wild asses of Little Rann Kutch, Gujarat
1970	HS	Killed 1500 Sambars in Sariska Tiger Reserve, Rajasthan
1973	Kyasanur	Killed 1808 Langurs and 315 Macaques in the Kyasanur Forest

	Forest Disease	of Karnataka
1980	RP	Caused mortality among Wild buffaloes of KNP, Assam
1980	HS	Killed 12 Rhinos in KNP, Assam
1987	FMD	Affected Wild buffaloes of KNP, Assam
1999	HS	Killed 3 Wild buffaloes in KNP, Assam
1999 onwards	Pesticides & unidentified etiology	Vulture population decreasing all over India

One of the major problems with the use of captive-bred populations for reintroduction into the wild (or the supplementation of depleted wild populations) is the spread of disease to non-infected populations. Attempts to return captive desert tortoises back to their native habitats backfired when released captives spread a respiratory disease to the wild populations. Often, populations of animals resistant to local disease organisms are used to establish captive breeding programs for supplementation of wild populations. When released into area where the disease organism is non-endemic, non-resistant populations are often decimated by the exotic disease.

Health management of wild animals:

In conservation programme of wildlife, health monitoring and disease investigation must be an essential component. There are several disease management and control techniques for the purpose like (1) Quarantine, (2) Isolation, (3) Test and slaughter, (4) Ring vaccination/Barrier vaccination of domestic population, (5) Immunization of wild animals, (6) Mass treatment of wild animals, (7) Deworming drugs etc.

People might belief that the free ranging wild animals posses a high standard of health and they, therefore, do not suffer from diseases. But the facts remain that they may possess a high standard of health but they too become victims of diseases. Disease is a major decimating factor of wild fauna. The free ranging wild animal no longer live in pristine environmental condition and that activity of human beings directly or indirectly affect every wild species which result in disease and which, therefore, must be managed to prevent the wild animal population from being impacted upon adversely by the deleterious effects of the disease.

The role of veterinarians and veterinary experts is enormous in health management of wild animals which includes treatment of sick and injured in wild and domestication, health monitoring of wild animals, immunization of livestock in and around forests, control of problematic animals particularly elephants in musth, tranquilization and translocation of wild elephants, transportation of wild and domesticated animals like elephants, post mortem and forensic support, registration of elephants (by microchipping), insurance of domesticated elephants, ecodevelopment on the fringe of the wild animal habitats through livestock improvement and fodder development etc. It is also necessary to identify unnecessary cruelties to these animals and developing humane methods to handle them both in wild and captivity.

Database on wildlife diseases:

Database on wild life diseases both for natural habiatats and captivity is not readily available in India, which is necessary to formulate strategies for control of the diseases. The basic information on incidence of diseases, mortality/causes of mortality (main/supplementary information) records on natural death (old age), trauma / infighting, drowning (flood), predation, poaching, (pit, Gunshot, electrocution), road accident, disease (with P.M. report) etc. are required to be maintained. However, records on such information in different species of wild animals maintained under captivity in different zoos of northeastern region have been analyzed under the guidance of the author for

preparing health management programme in those zoos.

The preventive health care information for wild life should also include information on domestic animals around the protected areas, information on disease out break along with mortality, morbidity and pathology in domestic animals in fringe areas, information on ecological relationship, information on various methods of immobilization, information on health assessment measures, zoonotic diseases etc. and information on disease control measures/vaccine efficacy etc. Information generation and dissemination, specific to a particular region and wild animal on the followings are required for disease control and prevention:

(a) Habitat requirements,
(b) Housing for captive animals,
(c) Feeding standards,
(d) Stress management in captivity,
(e) Preventive measures developed to check trauma,
(f) Solution to predator problems,
(g) Measures to check inbreeding,
(h) Immunization response,
(i) Control of infectious, parasitic diseases etc.,
(j) Control of zoonatic diseases, zoo personnel health and hygiene, and
(k) Experience sharing in necropsy studies and carcass disposal.

Information sharing on following disease management techniques is also required essentially to adopt control measures:

(a) Ring vaccination/ Barrier vaccination of domestic livestock (FMD, HS, Anthrax etc.),
(b) Response to immunization by wild animals: (Against Rabies by use of Oral Rabies Vaccine),
(c) Elimination of the causal agent,
(d) Mass treatment of wild animals,
(e) Control of insect vectors,
(f) Animal dispersal technique,
(g) Selective removal of diseased animal,
(h) Reduction of population density

Information Technology (IT) applications are changing our society and the world. The veterinary and wildlife profession is no exception. Therefore modernization of information generation and sharing is required to be made in wild life management and conservation by application of various newer technologies.

Many believe that disease management in free-ranging wild animals is not possible. But in fact it is possible to manage disease in free-ranging wild animals by manipulating the environmental factors that play a role in the transmission of disease.

METAGENOMICS: A GENOMIC TOOL FOR MICROBIAL DIVERSITY

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Introduction

Metagenomics term was first used by Jo Handelsman, referred as collection of gene sequenced from the environment could be analyzed in many analogies to the study of a single genome. In simple words we can say that collection of microbial gene from the environments without use of isolation and culture of individual microbes, by passing the need for isolation and lab cultivation of individual species. Most of the microorganisms are uncultured in the laboratory (about 99%) only 1% cultured in the lab. This technique useful for that huge number of microorganisms which are unculturated. Metagenomics works carried out in ocean, sewage, soil, human / animal gut micro-organisms; air and water microorganisms. The first report on an environmental library construction dates back to 1991 (Schmidt *et al.*, 1991). Metagenomics is the culture-independent analysis of a mixture of microbial genomes (termed the metagenome) using an approach based either on expression or on sequencing. Recent studies in the Sargasso Sea, acid mine drainage, soil, and sunken whale skeletons have used the shotgun-sequencing approach to sample the genomic content of these varied environments. In each study, environmental samples were obtained and the microbial DNA was extracted directly from the sample, sheared, cloned into Escherichia coli, and random clones were sequenced. In some of the studies sequence overlaps were then used to assemble contigs or scaffolds of genomic sequence.

According to this several synonyms for the metagenomic approach circulate in the literature: environmental DNA libraries (Stein *et al.*, 1996), soil DNA libraries (MacNeil *et al.*, 2001), eDNA libraries (Brady and Clardy, 2000), recombinant environmental libraries (Courtois *et al.*, 2003), community genome (Tyson *et al.*, 2004), whole genome shotgun sequencing (Venter *et al.*, 2004), environmental genomics (Delong *et al.*, 1999). There are several attempts to define what metagenomics is? The first is more technical whereas the second and third are more general in defining the aim of the metagenome approach independent of the techniques used.

Definitions.

1. Metagenomic libraries are databases of bacterial clones, usually Escherichia coli, carrying DNA fragments that originate from the collective genomes of all organisms present in a particular environment, habitat or assemblage" (Leveau *et al.*, 2004).
2. Metagenomics describes the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample" (Riesenfeld *et al.*, 2004).
3. The metagenome approach is the culture independent genomic analysis of microbial communities in the environment" (Riesenfeld *et al.*, 2004).

Aims of metagenomics

1. Study of phylogenetic diversity by using 16s rRNA
2. Study of beneficial enzyme for humen society eg. cellulases, chitinase, antibiotics and natural products.
3. Study of variation within genes for important enzymes that provides guidance for the designing the catalysts.
4. Single transduction of desired enzyme.
5. Plasmid/ Bacteriophage (vector) sequence analysis.
6. Knowledge of Genetic plasticity.
7. Study of metabolic pathways of microorganisms which helpful in designing in culture media
8. Identification of marker genes.

APPROACHES TO METAGENOMIC ANALYSIS

Metagenomic analysis involves isolating DNA from an environmental sample, cloning the DNA into a suitable vector transforming the clones into a host bacterium, and screening the resulting transformants. The clones can be screened for phylogenetic markers or "anchors," such as 16S rRNA and recA, or for other conserved genes by hybridization or multiplex PCR or for expression of specific traits, such as enzyme activity or antibiotic production or they can be sequenced randomly. Each approach has strengths and limitations; together these approaches have enriched our understanding of the uncultured world, providing insight into groups of prokaryotes that are otherwise entirely unknown.

Random sequencing

In a random sequencing approach, the clones are randomly chosen and end-sequenced, and the resulting sequences are assembled into larger contiguous pieces (contigs) by matching up overlapping sequences. The resulting data are contigs of different lengths as well as shorter unassembled fragments. The availability of completely sequenced "reference" genomes may assist in the assembly process for closely related genomes.

Targeted sequencing

Clones are first screened for the presence of a desirable gene (e.g., by PCR amplification) or a gene function (by functional assay). Sequencing targeted large-insert clones in their entirety allows the possibility of recovering complete operons, e.g., those encoding metabolic pathways. A common approach is to target fosmids bearing phylogenetically informative genes such as 16S rRNA. In this method, known as Phylogenetic Anchoring, if a 16S rRNA gene is detected, the fosmid insert is sequenced in its entirety, allowing us to assign the genomic DNA sequence to a specific phylotype. So, unlike random sequencing, this approach helps affiliate phylogeny (rRNA) with putative functional genes (predicted from flanking insert sequences), and permits us to confirm/dispute old assumptions of "who does what." A classic example of this was the discovery of rhodopsin-like photoreceptor genes (light-driven proton pumps for energy production) in Monterey Bay BAC clones harboring 16S rRNA genes (Aravind *et al.* 2000). Where previously rhodopsins (and rhodopsin-based phototrophy) were thought to be exclusively archaeal in origin, metagenome data from phylogenetically anchored clones indicated that this functionality exists in marine g- and a-proteobacteria as well (Beja *et al.*, 2000; Sabehi *et al.*, 2004; Sabehi *et al.* 2005). Thus,

metagenomics is emerging as a powerful method to study the function and physiology of the unexplored microbial biosphere, and is causing us to reevaluate basic precepts of microbial ecology and evolution. Fosmids bearing process-specific or biomarker genes (e.g., for processes that may be prominent in the environment under study, like methane oxidation or denitrification) may also be targeted for sequencing to expand information on pathways for these processes.

Functional Metagenomics

Heterologous expression

A powerful yet challenging approach to metagenomic analysis is to identify clones that express a function. Success requires faithful transcription and translation of the gene or genes of interest and secretion of the gene product, if the screen or assay requires it to be extracellular. Functional analysis has identified novel antibiotics (Courtois *et al.*, 2003; Gillespie *et al.*, 2002; MacNeil *et al.*, 2001; Venter *et al.*, 2004; Wang *et al.*, 2000), antibiotic resistance genes (Diaz-Torres *et al.*, 1993; Riesenfeld *et al.*, 2004), Na+(Li+)/H+ transporters (Majernik *et al.*, 2001), and degradative enzymes (Healy *et al.*, 1995). The power of the approach is that it does not require that the genes of interest be recognizable by sequence analysis, making it the only approach to metagenomics that has the potential to identify entirely new classes of genes for new or known functions. The significant limitation is that many genes, perhaps most, will not be expressed in any particular host bacterium selected for cloning. In fact, there is an inherent contradiction in this approach, genes are cloned from exotic organisms to discover new motifs in biology, and yet these genes are required to be expressed in Escherichia coli or another domesticated bacterium in order to be detected. The diversity of the organisms whose DNA has been successfully expressed in E. coli is surprising, but heterologous expression remains a barrier to extracting the maximum information from functional metagenomics analyses.

Identifying active clones, screens, selections, and functional anchors

The frequency of metagenomic clones that express any given activity is low. For example, in a search for lipolytic clones derived from German soil, only 1 in 730,000 clones showed activity (Henne *et al.*, 2000). In a library of DNA from North American soil, 29 of a total of 25,000 clones expressed hemolytic activity (Rondon *et al.*, 2000). The scarcity of active clones therefore necessitates development of efficient screens and selections for discovery of new activities or molecules. Just as bacterial genetics relies on selections to detect low-frequency events, metagenomics will be advanced by seeking selectable phenotypes to increase the collection of active clones that can be compared, analyzed, and used to build a conceptual framework for functional analysis. Several selections have proved to be fruitful. For example, the Daniel group designed a clever selection for Na+(Li+)/H+ antiporters that requires complementation of an E. coli mutant deficient in the three Na+(Li+)/H+ antiporters (nhaA, nhaB, and chaA) enabling growth on medium containing 7.5 mM LiCl (Majernik *et al.*, 2001). This powerful selection facilitated the discovery of two novel antiporter proteins in a library of 1,480,000 clones containing DNA isolated from soil. Another selection strategy involved complementation of an E. coli mutant deficient in biotin production, which led to the isolation of seven new operons for biotin synthesis from enrichment cultures derived from samples of soil or horse excrement (Entcheva *et al.*, 2001). Selection for antibiotic resistance led to the isolation of a tetracycline resistance determinant from samples of the microbiota from the human mouth (Diaz-Torres, *et al.*, 2003) and aminoglycoside resistance determinants from soil (Riesenfeld *et al.*, 2004). The selection for aminoglycoside resistance identified nine

clones, six of which encoded 6-acetyltransferases that formed a new cluster based on sequence analysis. These genes were discovered in libraries containing a total of 4 Gb of DNA, or approximately 1 million genes, and thus their infrequent representation would have made it prohibitively laborious to discover them by a screen without a selection. This example illustrates the power of functional metagenomics, genes that are expressed in an ordinary host such as E. coli may be extraordinary and novel. High-throughput screens can substitute when the functions of interest do not provide the basis for selection. For example, on certain indicator media, active clones display a characteristic and easily distinguishable appearance even when plated at high density. With the indicator dye tetrazolium chloride, Henne *et al.*, 1999 detected clones that utilize 4-hydroxybutyrate in libraries of DNA from agricultural or river valley soil. Very rare lipolytic clones in the same libraries were detected by production of clear halos on media containing rhodamine and either triolein or tributryin (Henne *et al.*, 2000). The discovery of new biological motifs will depend in part on functional analysis of metagenomic clones. Functional screens of metagenomic libraries have led to the assignment of functions to numerous "hypothetical proteins" in the databases. Innovation will be required to identify and overcome the barriers to heterologous gene expression and to detect rare clones efficiently in the immense libraries that are needed to represent all of the genomes in complex environments, such as soil. An emerging and powerful direction for metagenomic analysis is the use of functional anchors, which are the functional analogs of phylogenetic anchors. Functional anchors are functions that can be assessed rapidly in all of the clones in a library. When a collection of clones with a common function is assembled, they can be sequenced to find phylogenetic anchors and genomic structure in the flanking DNA. Such an analysis can provide a slice of the metagenome that cuts across clones with a different selective tool, determining the diversity of genomes that contain a particular function that can be expressed in the host carrying the library. Technological developments that promote functional expression and screening will advance this new frontier of functional genomics.

Sequence-based screening for small molecules

The first polyketide synthases, enzymes involved in synthesis of polyketides, the broad class of antibiotics that includes erythromycin, epithilone, and rifamycin, were first cloned from soil with a PCR based approach. Seow *et al.*, 1997 designed primers that hybridize with the highly conserved region of polyketide synthase genes and amplified novel polyketide synthase homologues directly from soil. This approach was adapted for screening metagenomic libraries by Osburne's group, who screened a 5,000-member metagenomic library for conserved regions of genes encoding type I polyketide synthase. Primers directed toward a conserved region of polyketide synthase I genes that flanks the active site of the ketoacyl synthetase domain were used to screen pools of 96 clones. The screen yielded 11 new polyketide synthase homologues that contained significant sequence similarity to polyketide synthase genes from cultured organisms. In addition, screening clones in both E. coli and Streptomyces lividans by chemical means revealed two novel compounds, fatty dienic alcohol isomers.

Antibiotics as signal molecules

The study of interspecies microbial interactions mediated by antibiotics is as old as the science of microbiology itself. Such studies remain particularly relevant today because of our current struggles to deal with failures of antibiotic therapies. By integrating new technologies and scientific concepts, such as metagenomics and sociomicrobiology into microbiology, we are learning how to define the structure and behavior of microbial communities in a way that was not previously pos-

sible. The study of microbial interspecies interactions can thus shed light not only on how microorganisms colonize hosts or particular environmental niches, but also on what roles polymicrobial communities play in health and disease.

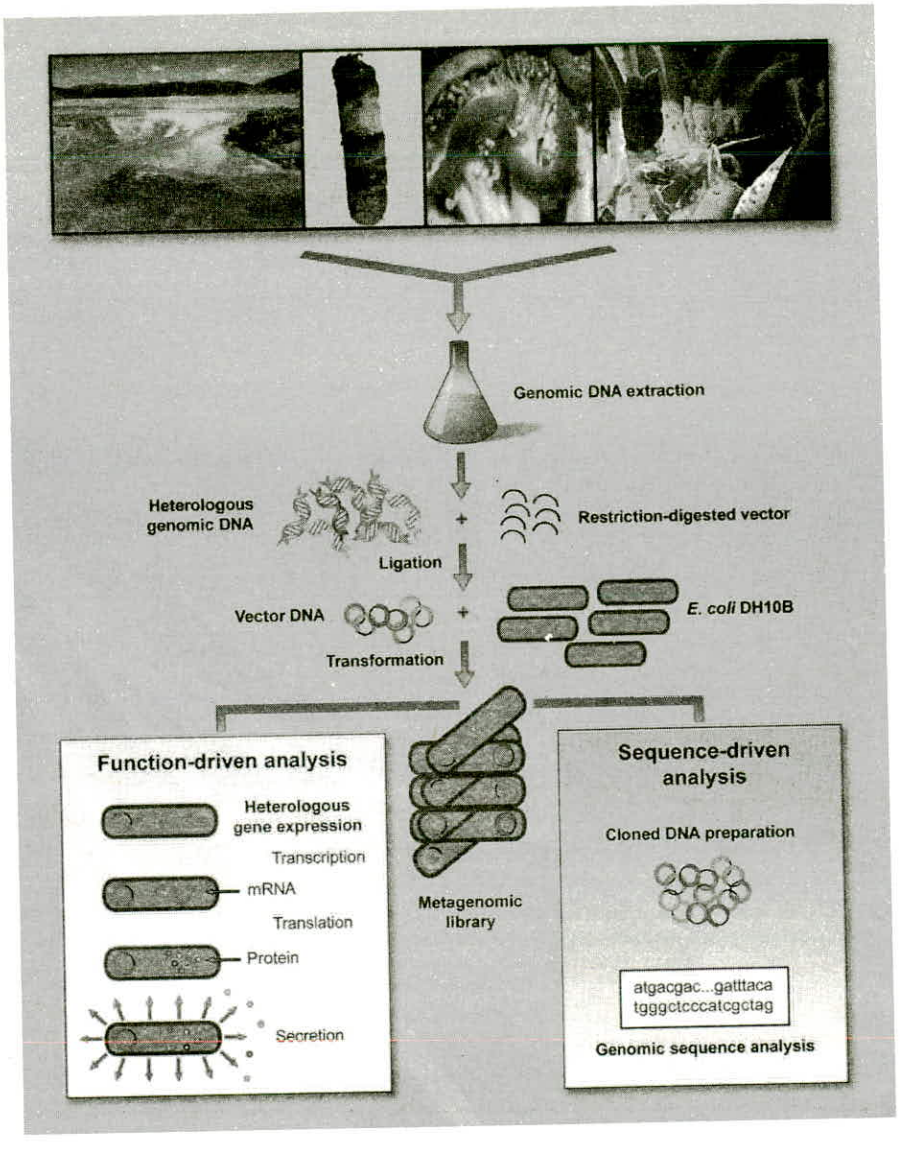

Fig: Schematic representation of construction of Metagenomic libraries

From such a perspective, antibiotics are likely to play multiple natural roles, ranging from communication to manipulation to warfare. An improved understanding of these natural roles can inform the therapeutic use of antibiotics, with the hope of explaining or even predicting clinical treatment failures. For instance, microbes are able to sense and respond to structurally diverse antibiotics

through specific signaling pathways, leading to resistance that is often transient and thus not detected by routine testing. Thanks in part to genome sequencing, there is increasing recognition that many signaling mechanisms by which microbial cells sense and respond to antibiotics are highly conserved. Despite such conservation, these systems are integrated into the physiologies of particular organisms in myriad ways, contributing to the extraordinary diversity of the microbial world.

If antibiotics evolved as mediators of functions other than warfare (Davies *et al.*, 1990), such as communication, antibiotic discovery will be expedited by screening metagenomic clones for signaling compounds as well as inhibitory compounds. The challenge is to develop assays that detect signaling by many compounds. A surprising result from the Davies group indicated that sub inhibitory concentrations of many antibiotics induce quorum sensing despite no resemblance in structure to the acylated homoserine lactones that appear to be the natural inducers (Goh *et al.*, 2002). This result presents a propitious opportunity a single screen might capture molecules that are quorum-sensing inducers as well as antibiotics. This opportunity was investigated by designing a high throughput screen to identify compounds that induce the expression of genes under the control of a quorum-sensing promoter. The screen is intracellular; meaning the metagenomic DNA is in the same cell as the sensor for quorum-sensing induction. The sensor is comprised of the luxR promoter, which is induced by acylated homoserine lactones, linked to gfp, and resides on a plasmid in an E. coli strain that did not induce quorum sensing itself (Andersen *et al.*, 2001). If an inducer of the luxR-mediated transcription of gfp is expressed from the metagenomic DNA, the cell fluoresces and can be captured by fluorescence-activated cell sorting or as a colony observed by fluorescence microscopy. Conversely, this sensor system can detect inhibitors of quorum sensing if acylated homoserine lactone is added to the medium and fluorescence-activated cell sorting is set to collect the nonfluorescent cells. Metagenomic libraries from microbiota of the soil and from the midgut of the gypsy moth have been subjected to this screen, and arrays of genes have been identified. Their products are under analysis, and some appear to differ from previously described quorum sensing inducers.

CONCLUSIONS AND FUTURE DIRECTIONS

Metagenomics has changed the way microbiologists approach many problems, redefined the concept of a genome, and accelerated the rate of gene discovery. The potential for application of metagenomics to biotechnology seems endless. Functional screens have identified new enzymes and antibiotics and other reagents in libraries from diverse environments. A number of barriers have limited the discovery of new genes that provide insight into microbial community structure and function or that can be used to solve medical, agricultural, or industrial problems. Realizing the potential for discovery from metagenomics is dependent on the advancement of methods that are central to library construction and analysis. Sequence-based assignment of function will also benefit from advances in detection of homology, which will increasingly rely on the tertiary structures of predicted proteins rather than simply on primary sequence. Advances that will facilitate the management and analysis of large libraries include bioinformatics tools to analyze vast sequence databases and reassemble multiple genomes rapidly and affordable gene chips for library profiling or those readily distinguish clones that are expressing genes from those clones that are silent. Functional analysis will require more innovation in method development. Most important among these are strategies to improve heterologous gene expression and approaches for efficient screening of large libraries. Microbiology has long relied on diverse methods for analysis, and

metagenomics can provide the tools to balance the abundance of knowledge attained from culturing with an understanding of the uncultured majority of microbial life. Myriad environments on Earth have not been studied with culture-independent methods other than PCR-based 16S rRNA gene analysis, and they invite further analysis. Metagenomics may further our understanding of many of the exotic and familiar habitats that are attracting the attention of microbial ecologists, including deep sea thermal vents; acidic hot springs; permafrost, temperate, desert, and cold soils; Antarctic frozen lakes; and eukaryotic host organs-the human mouth and gut, termite and caterpillar guts, plant rhizospheres and phyllospheres, and fungi in lichen symbioses. With improved methods for analysis, funding stimulated by recent triumphs in the field, and attraction of diverse scientists to identify new problems and solve old ones, metagenomics will expand and continue to enrich our understanding of microorganisms.

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SESSION VI

Bioinformatics: Applications for development of Biologicals

Chairman : **Dr. B.D. Lakhchaura**

Co-Chairman : **Dr. Soma S. Marla**

Rapporteur : **Dr. P.K. Gupta**

LEAD PAPERS

VI.1 Nano-bio-information Technology: The Horizon of a New Concept in Life Science Research
Anil Kumar, Soma S. Marla, Dinesh Pandey and B. R. K. Gupta

VI.2 Pharmacogenomics : the new concept for the drugs of future
S.P.Singh and T. Ambwani

VI.3 Role of Biosafety and Biocontainment facility: Concerns and future Challenges
S.C. Dubey & H.V. Murugkar

ABSTRACTS

6.01 Sequence Analysis of a Major Outer Membrane Protein (ompH) Gene of Pasteurella multocida P52
Rashmi Singh and V.D.P. Rao

6.02 Molecular cloning and comparative sequence analysis of mammary gland and abomasal lysozyme from buffalo and sheep
Sudarshan Kumar, Pradeep M.A., Mohanty A.K. and Kaushik J. K.

6.03 Composite Proteins: Generating Non-Immunogenic Therapeutics
Neelofar Mirza, Sonu Ambwani and Anil Kumar

6.04 PCR based confirmation, *HSP65* gene sequencing and spacer oligonucleotide typing of *M. bovis* and *M. tuberculosis* isolates from cattle in Himachal Pradesh
Aneesh Thakur, Mandeep Sharma, Vipin Katoch and Prasenjit Dhar

NANO-BIO-INFORMATION TECHNOLOGY: THE HORIZON OF A NEW CONCEPT IN LIFE SCIENCE RESEARCH

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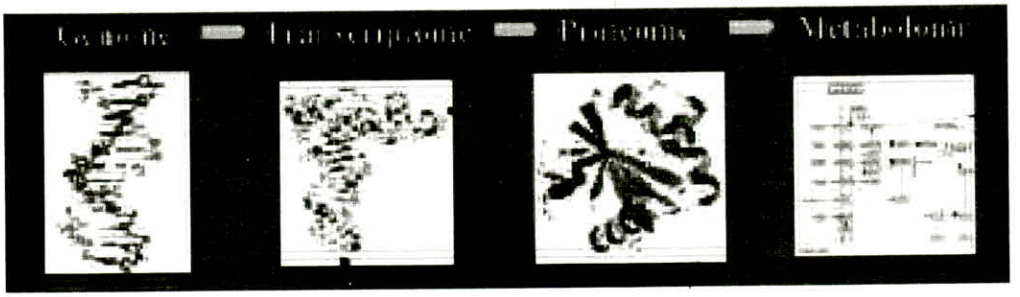
Successful realization of life science research depends on employing bioinformatics to unravel patterns in complex experimental data sets generated from genomics & proteomics research, precise technology for understanding the diagnosis and interventions designed at the nano-scale to develop specific as well as effective desirable product. New dimensions in life science research, in short, are being evolved by integration of biotechnology, nanotechnology and informatics. Bioinformatics and nano-bioinformatics have now emerged as separate disciplines, which serve as cohesive forces in binding these technologies together. Bioinformatics played a key role in handling and use of huge information generated through the explosive growth of Molecular biology and genomics. Nano-bioinformatics holds potential for the application of nanotechnology-based diagnostics and therapeutics for personalized medicine and health. The incredible sensitivity, flexibility and high throughput nanotechnologies combined with the modularity of nanoparticle based technology has poised to play a key role in medical, veterinary and agricultural applications as well as in industrial revolution.

In 21st century, research in life sciences has witnessed a shift from conventional to molecular approach for understanding the biological systems. Whereas conventional approaches were largely based on the analysis of system at phenotypic level, molecular approaches analyze it at genotypic level. This has unraveled the biological processes at molecular level, which has enabled scientists to identify useful biomolecules particulary genes/proteins, which can be precisely manipulated in a biological system so as to produce a desirable product. Moreover, there has been a paradigm shift from single gene approach (ie. Gene by gene approach) in understanding the mechanism of biological phenomenon to working with several genes at a time. This shift has resulted from the observation that any biological response results from cross talk of many genes, which act in interdependent manner in signal transduction pathways. The realization that any biological response results from convergence and integration of various molecular networks has led to a "holistic approach" to study the biological problems. As a result, many high throughput technologies have been evolved which provide a glimpse of all the molecules involved in a process. The process has been accelerated by research in the field of genomics. The data obtained from sequencing projects such as recently sequenced buffalo genome are extremely important for researchers in veterinary, agriculture and medicine to understand the functions of key genes & proteins involved in growth, development and imparting resistance to numerous diseases and unfavorable conditions. However there exists a wide gap between genotype and phenotype in manifestation of a trait. To fill this gap research is conducted at different levels -Whole plant, Cellular, biochemical, gene and protein levels. All these areas of unparalleled scientific efforts have led to the accumulation of large quantities of biological information. Nanobioinformation technology which is culmination of bioinformatics and nanotechnology attempts to use these data to design novel strategies to address the problems of agricultural, veterinary, medicinal and industrial importance.

Life Science Research in Post-genomic era

During the last decade genomes of Rice, buffalo, humans and several other genomes have been sequenced. Sequencing of genomes enables us to better understand the life of an organism. Huge data resulting from genome sequencing is analyzed to predict the protein coding genes; structure of genes and proteins, functions they perform and the relationships existing among them. Computer science has really helped Biology to perform the complex job of analyzing the enormous data and to reveal the encrypted information from sequenced genome. For example a daunting task performed by computers is in identification of the location for functioning genes in newly sequenced genomes (for example although human genome is 3,000 nucleotide mega bases in size, only 4 percent of it contains functioning genes that can produce proteins).

Research conducted in genomics and proteomics will need the skills and tools for organizing, interpreting, and storing biological information. There are many levels at which the sequenced genome information can be used, such as Transcriptomics, Proteomics, metabolomics (Fig.1), to develop predictive methods for studying the functioning of the phenotype of an organism. For example the data obtained from sequencing of Buffalo genome appears promising using the fields of genomics, proteomics.



Fig, 1 Life science research can be conducted at different levels of organization. (Source: United States dept. of Energy, 2000)

The available data from human genome project is likely to be of significant assistance in medical genetics especially in diagnosis of diseases & design of drugs. Detection of all the causative defective or abnormal genes inherited from the parents is not an easy task. Where as Genomics in medicine (making use of all available genetic information) has made important strides in diagnosing of genetic diseases by developed DNA probes for screening diseases even before they are not functional. Similarly, various proteomics tools such as protein arrays, 2.D gel separation and development of easily scorable protein markers from diseased tissue samples are enabling are transforming disease diagnosis an accurate and reliable task. Consequently, data recording from experimentation is gradually shifting from descriptive mode to digital form for making it suitable to use computers for data analysis and its meaningful interpretation.

Bioinformatics and *In silico* biology research

Bioinformatics is an interdisciplinary scientific area, which combines the sciences like mathematics, biology, computer science and information technology to collect, process and analyze the scientific data obtained through life science research and genomics. In recent years this

discipline has grown so much that the huge biological information has been presented in the form of numerous data bases which are specific for plants, animals, bacteria and human beings. These databases help in locating new genes and proteins which are useful for further research and in designing novel drugs as well as in smart drug delivery system.

Precisely, Bioinformatics can influence productivity of life science research in modern biology through following ways:

1. Development of databases: Designing user friendly databases for storing and easy retrieval of research information generated form different sources and laboratories help in making meaningful discoveries faster and efficiently. For example, animal germplasm databases are a wonderful resource for improvement of cattle breeds fro example.

2. Comparative genomics: The level of homology/variation among the species and presence of orthologs genes is possible by comparing given gene/protein sequences with the database which has deposited list of sequences from all other organisms.

3. Discovery of new coding gene: it is possible to locate genes coded for immunity expression of traits such as diseases by analyzing the raw sequence data and using sequence comparison techniques

4. Gene expression analysis, prediction of gene function and establishment of gene libraries at various growth stages or during progression of a disease (Functional Genomics)

5. Identification of proteins, detection of their structure and function by using their gene sequences

6. Modeling and simulation of a metabolism in both healthy and diseased organisms to better understand the disease mechanism.

7. Obtained knowledge from biochemical & metabolic pathway dynamics can help medical researchers in identification of genes/genomic regions associated with inherited diseases in animals and humans.

8. Data obtained from functional genomics (Micro array data from diseased and healthy tissue samples) could be used in drug designing and drug discovery.

Currently, bioinformatics approaches to deal with this problem involve designing search algorithms and tools using neural nets, Hidden Markov models or vector machines. Precisely discovery of new genes is viewed as a pattern recognition problem that employs apart from the probabilistic modeling techniques cited above also evolutionary computation techniques such as Genetic algorithms. For example time required for development of a new drug in pharmaceutical industry can be reduced from current 10 years period to 4 to 5 years by employing functional genomics techniques like Micro arrays(Fig.2) and Bioinformatics tools for drug design.

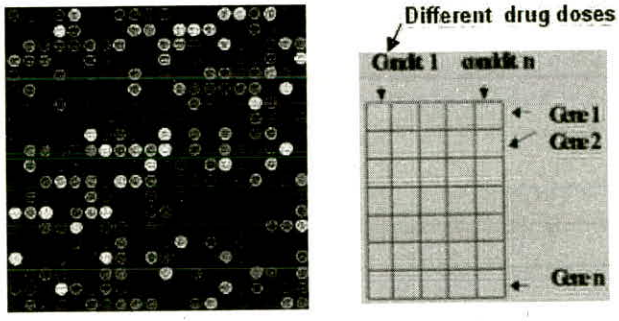


Fig.2: A simple Microarray and Expression Data Matrix

One of the major issues in management of Animal Genetic stock improvement programs is management of germplam and biodiversity information. Bioinformatics databases designed with dynamic content could aid animal breeders in their breeding programs.

Bio-informatics in health & diseases

Medical practice is changing at a rapid pace due to the influx of biological information from novel technologies. The management and analysis of this information is critical to the development of personalized medicine. For example, informatics can be applied to the identification of single nucleotide polymorphisms that may correlate with disease, disease susceptibility, or adverse drug or stress reactions. In addition, many important medical conditions and risk factors are multigenic or, as a further complication, depend on interactions between gene products and the patterns of their expression and post-translational modification and, therefore, can also benefit from informatics tools. An example how bioinformatics tool can be exploited to design a new drug that binds human target protein is illustrated in fig.3. Bioinformatics tools help in locating and predicting the structure of target protein by comparing it with similar sequences known in model organisms. Once the structure is modeled, its interaction with drug can be studied using bioinformatics softwares.

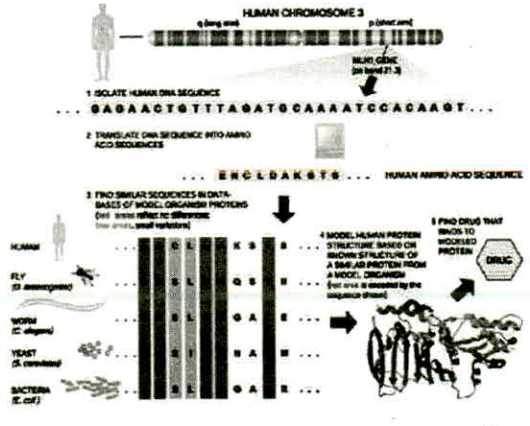


Fig. 3. Illustration of how bioinformatics help in improvement in Human health starting from disease gene discovery to drug development.

Nanotechnology: Potential theme of research

Nanotechnology is a modern area of science, which includes the manipulation, or self-assembly of individual atoms, molecules, or molecular clusters into structures to create materials and devices with new or vastly different properties. Nanotechnology can work from the top down (which means reducing the size of the smallest structures to the nanoscale e.g. photonics applications in nanoelectronics and nanoengineering) or the bottom up (which involves manipulating individual atoms and molecules into nanostructures and more closely resembles chemistry or biology). It is, infact, the convergence of technology with Molecular Biology at nanoscale. Nanotechnology has the enormous potential to boost research efforts in basic, life, agricultural and veterinary sciences. Major applications of nanotechnology include nanocoating, nucleic acid bioengineering, smart treatment delivery systems, nano-bioprocessing, bio-analytical nano-sensors, nano-materials and bio-selective surfaces which have applications in every field of science. For example development of low cost sensors for modern diagnostics can detect the presence of food borne and agriculturally important pathogens, filters for removing undesirable compounds from foods and beverages and also nano-particles to store flavors in meat and other packaged foods. With advent of Hi-throughput experimentation technologies such as DNA & Protein chips (Micro arrays) nanotechnology has the potential to miniature and represents a virtual plant or animal cells. This can aid in better understanding of various cellular processes mechanisms regulating important agronomic traits and may contribute to the improvement of products in agriculture such as pest & disease resistant crops with high nutrition value. It's broad spectrum applications in the areas of bio-medical science include gene transfection, targeted drug delivery, diagnosis and treatment of various severe diseases like HIV, cancer and genetic disorders

The combination of "Biotechnology" and "Nanotechnology" would likely to result in development of advanced transport phenomena and release mechanisms by various forms of physical preparation or chemical synthetic nano particles and nano tubes. The ultimate goals will be the practices of these innovations for the diagnostic and therapeutic in clinics (e.g. gene therapy, early cancer detections and treatments, organ replacement, metabolic pathway analysis and free radicals detections, etc), the agricultures (GMO) and environment (pesticides). In doing so, we will certainly help to bring better living quality for the all the human beings. More recently, nanotechnologies are predicted to have a radical impact on how we study, diagnose and treat disease. Researchers develop and apply nanotechnology to all aspects of drug development and patient care from streamlining the drug development process through miniaturization, increased sensitivity, high-throughput analysis and automation for target identification and validation; developing quantum dots for in vivo imaging; utilizing contrast and optical imaging agents and ultra sensitive biomarker detection for early detection and diagnosis; to developing a plethora of multifunctional, targeted drug delivery vehicles for treatment and, eventually, real-time therapeutic monitoring.

Nanotechnology has been described as the new industrial revolution and both developed and developing countries are investing in this technology to secure a market share. At present the USA leads with a 4 year, 3.7 billion USD investments through its National Nanotechnology Initiative (NNI). The USA is followed by Japan and the European Union, which have both committed substantial funds (750 million and 1.2 billion, including individual country contributions, respectively per year). The level of funding in developing countries may be comparatively lower, however this has not lessened the impact of some countries on the global stage. For example, China's share of academic publications in nanoscale science and engineering topics rose from 7.5% in 1995 to 18.3% in

2004, taking the country from fifth to second in the world. Others such as India, South Korea, Iran, and Thailand are also catching up with a focus on applications specific to the economic growth and needs of their countries. Iran for example has a focused programme in nanotechnology for the agricultural and food industry. A recent study from the Helmuth Kaiser Consultancy predicts that the nanofood market will surge from 2.6 billion USD to 20.4 billion USD by 2010. The report suggests that with more than 50% of the world population, the largest market for Nanofood in 2010 will be Asia lead by China.

Nano-bioinformatics: The Enabling Technology of Personalized medicine & Health

Nanobioinformatics is an emerging field of study born out of a necessity of nanotechnology researchers in the life sciences whose work is dependent on timely access to an emerging wealth of data. It brings together the three very diverse fields of nanotechnology, molecular biology and biomedical informatics that until now have had little overlap and where major cultural gaps exist. It is critical that nano-bioinformatics be embraced as an extension of biomedical informatics and not be orphaned prematurely. Because of the combinatorial nature of nanoparticle composition (core + surface modifier + payload) and the enthusiasm for this area in general, nanotechnology in biomedicine will see a rapid exponential increase in the data produced, thus making it critical to develop robust infrastructure to support data standardization and storage, modeling and simulation, symbolic reasoning and information retrieval. The foundation of this infrastructure is the development of ontologies and databases of the description and physical characterization of the nanoparticle-based diagnostics and therapeutics. Systematically identifying and analyzing essential information pertaining to physical, chemical, structural, mechanical, biological and other parameters is critical to understanding structure-function relationships and resultant medical applications and to making this information accessible to the clinical community. For instance, there are already examples of multifunctional nanoparticles that target vascular peptides, growth factor receptors, and transmembrane proteins such as ion channels that are utilized for both cancer and cardiovascular disease recognition.

Current status of nano-bioinformation research

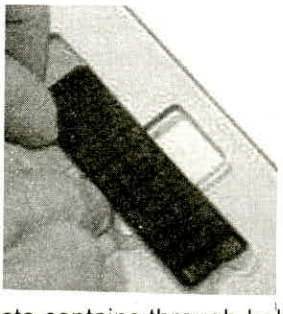
Comparing to the current progress of international efforts on bio-nanotechnology, we are not only short of human resources and financial supports but also lack integration and communications. Even though the current efforts of nanotechnology are more concentrated on the engineering applications, nevertheless nature has been master in constructing elements of life from nano-sized molecules such as proteins, nuclides, hydrocarbon, lipids etc. Together with the fundamental knowledge regarding nano devices, materials drug design and manufacture. It would be feasible to directly manipulate or interface to the host for both diagnosis and therapy. "BIONANO" root in biophysics and biochemistry and have many relevant technologies in synthesis, assembly, genomics, proteomics, structural biology, toxicology, theoretical biology, biomaterials, bioelectronics, bioinformatics, and biochips. For molecular level, we have biochip for disease detection, recognition mechanisms of nano structure, lipid polymer for drug targeting. At cellular level, we have nano-crystal for fluorescent imaging, immune latex particles. At tissue level, we have multi-channel SQUID for magnetic imaging, nano vaccine, and drug delivering. For clinical applications, we have cardiovascular disease diagnostic kits and stent surface material, corneal membrane for regeneration. Each will focus on the novelty for academic excellence and feasibility for industrial mass production. The intended applications include cancer, footmouth disease, and gene-modified organics (GMO), which all have nano-scale limits. The periodic structure of nanometer multilayer

SPR can be used for unique optical detection mode will target portable device for personal diagnostic tool. Signal amplification scheme will also adapt for nanoparticle application. Many of the research results potentially can lead to commercial success.

The several research projects are going on which include

- Molecular recognition of biomolecules versus Nanostructures: Design, synthesis, Characterization and Applications
- Inorganic nano particles as optical catalytic agent
- Nano vaccine delivery system for gene therapy
- Nano magnetic immune latex particles
- A Study of Purified Montmorillonite Intercalated 5-ASA Composite for Inflammatory Bowel Diseases
- Bifunctional group copolymer as drug delivery system
- Biodegradable Nano capsule as a carrier for water soluble drug
- Cardiovascular disease screening and surface modification for STENT
- Infectious keratitis and corneal tissue regeneration by nano stacking
- Rapid nuclide screening kit for colon-rectal cancer
- Data mining for proteomic biochips
- Modified Au nano particles with Rolling circle amplification (RCA)
- Protein chip and multifunction optical reader
- Nano scale force mapping for proteomics
- Design, fabrication and applications of biological nano switch
- Fluorescent nano crystal imaging for functional cell studies
- Multichannel quantum dot for SQUID measurement
- Ecological monitoring by nanobiotechnology

All these applications of Nanotechnology have resulted in development of advanced high-throughput nano-devices, which have great flexibility, sensitivity.



The OpenArray™ plate contains through-holes for 3072 assays.

Researchers can test one to 48 different samples against a wide array of assays. The OpenArray™ SNP Genotyping system enables flexibility in experimental design in many research

areas: Candidate gene studies, Fine mapping, Pharmacogenomics, Linkage mapping, Allele specific transcript detection, Admixture mapping, Clinical diagnostics, Forensics, Genetic identification, Pathogen detection/ Biodefense, Animal husbandry, Crop science

At this stage of development, the research focuses on the molecular levels: cancer biochips, biomolecules/nano structures recognition, and micro-encapsulation drug delivery system; cellular level: nanoparticles; tissue level: quantum dot for multi-channel SQUID, immunizations; clinical level: cardiac vascular disease and surface modifications of biomaterial; and the final area: agriculture

The goals of this area include:

a. The comprehensions of nano devices within biomolecules/cell and their structure and functions;

b. The establishment and improvement of design and manufacture protocols, material bulk and surface properties;

c. The interaction model of device/biological, force, distance, migration rate, etc;

d. The applications for biomedical diagnostic, which include gene chip and protein chip system, bio-optics, implantable micro devices;

e. Development of clinical and biotechnological applications: drug delivery, microactuator, biomotor;

f. Environmental and agricultural applications: soil and water molecular detection, biomarkers, organic product monitoring system

Efforts at Pantnagar

Since the Nanoscience and Nanotechnology has emerged as one of the most innovative science these days which may also be run in our University in the interest of newly carved Uttarakhand state to provide the quality human resources. This also includes designing of Nanobiosensor, nanocoating, Nanofiltration and several nanotechnologies- based applications in almost all fields of Agricultural, Lifescience and Engineering sciences. Eventually, it may also open several opportunities for better management of agro industries as well. The M.Sc. programme in Nanoscience and Nanotechnology has been recently approved to run an inter-disciplinary programme in the College of Basic Sciences and Humanities under coordinator ship of Prof. B.R.K. Gupta, Dean, CBS&H. Hon'ble Vice Chancellor has further visualized a great need for opening a new branch of basic science i.e. 'Nanoscience and Nanotechnology' to keep pace with the modern research and developmental activities linked with Agricultural and Animal sciences in years to come. The current efforts of faculty members of different departments of the college are given below:

- Design of different types of nano-materials/nanopolymers for various usage
- Use of colloidal gold technologies in commercialization of immunotechnologies in the form of rapid diagnostic kits for detection of plant and animal diseases
- Use of colloidal sol in enhancing the agricultural productivity as plant growth stimulants
- Design of biosensors for agricultural applications.

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PHARMACOGENOMICS : THE NEW CONCEPT FOR THE DRUGS OF FUTURE

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INTRODUCTION

Individual genetic mapping will soon become widely affordable:

" The good news, however, is that a whole bunch of companies in the United States, Europe and elsewhere are now racing to reduce the cost and time factor involved. Basically, they are aiming to score in a global gene sequencing market which analysts expect to reach over $ 1 billion in the next two to three years. To this end, the US Government already committed grants totalling $ 56 million for the development of technology that could put large scale genome sequencing within everyone,s reach." (Times of India, 18 Feb., 2008)

The term pharmacogenomics has been derived origin from the words pharmacology and genomics and refers to the study of impact of genetic inheritance of an individual on response to drugs. Pharmacogenomics elucidates the inherited nature of intra-species variation in drug disposition and effects and thus provides a stronger scientific basis for selecting the optimal drug therapy and dosages for patient within the species. Pharmacogenomics holds the promise that one day drugs might be designed and produced genetically suitable to each individual's own genetic makeup. Understanding an individual's genomic composition and sequencing is thought to be the key for creating personalized drugs with greater efficacy and safety. Pharmacogenomics borrows from the traditional pharmaceutical sciences such as biochemistry with annotated knowledge of genes, proteins, and single nucleotide polymorphisms (SNPs) and thus is likely to influence the nature of drug discovery and drug delivery system.

ANTICIPATED BENEFITS OF PHARMACOGENOMICS

Individual based drugs and dosage regimen

The genomic based drug therapy will be more selective and targeted to specific diseases in certain individuals. Drugs will be discovered, and designed, and manufactured based on the targeted proteins, enzymes, and RNA molecules associated with genes and diseases and will be prescribed to individuals with matched genomic sequencing. Clinicians will be able to analyse and match patient's genetic profile and accordingly prescribe the best available drug therapy. It will speed up early medication with right drugs with the prospects of fast recovery. With an increase in safety level of the drug the likelihood of adverse reactions will be eliminated. Dosage regimens of drugs will also be more accurate as it will be based on an individual's genome which will greatly influence the pharmacokinetic trend of that individual for a particular drug that is how much time it takes to metabolize and excrete it. This will help accurately to maximize the therapy's value and decrease the likelihood of untoward effects of overdosing. Pharmacogenomics thus will have potential to dramatically reduce the estimated death and hospitalizations that occur each year due to metabolic, non-metabolic, infectious and many acquired and hereditary diseases in man and animals through out the world (Lazarao, et a., 1998).

Early diagnosis and care of disease

Pharmacogenomic approach will allow a person to make adequate lifestyle and environmental changes at an early age so as to avoid or lessen the severity of a genetic disease. Likewise, advance knowledge of susceptibility of a disease will allow careful monitoring, and treatments can be introduced at the most appropriate stage to maximize their therapy.

More effective vaccines

Vaccines prepared from, either DNA or RNA, promise all the benefits of existing vaccines without risks. They will activate the immune system but will be unable to cause infections. They will be inexpensive, stable, easy to store, and capable of being engineered to carry several strains of a pathogen at once.

Rapid drug discovery with minimum toxicity

Pharmaceutical companies will be able to discover potential therapies more easily using genome targets. The cost and risk of clinical trials will be reduced by targeting only those persons capable of responding to a drug. Genomic based drugs will have no risk of allergic, idiosyncratic and residual toxicity. The cytochrome P450 (CYP) family of liver enzymes is responsible for breaking down more than 30 different classes of drugs. DNA variations in genes that code for these enzymes can influence their ability to metabolize certain drugs. Less active or inactive forms of CYP enzymes that are unable to break down and efficiently eliminate drugs from the body can cause drug overdose in patients. Today, clinical trials researchers use genetic tests for variations in cytochrome P450 genes to screen and monitor patients. In addition, many pharmaceutics screen their chemical compounds to see how well they are broken down by variant forms of CYP enzymes (Hodgson and Marshall, 1998).

The enzyme thiopurine methyltransferase(TPMT) plays an important role in the human chemotherapeutics of infant's leukemia by breaking down thiopurines. A small percentage of Caucasians have genetic variants that prevent them from producing an active form of this protein. As a result, thiopurines elevate to toxic levels in the patient because the inactive form of TMPT is unable to break down the drug. Today, a genetic test is at hand of physicians to screen patients for this deficiency, and the TMPT activity is monitored to determine appropriate thiopurine dosage levels (Pistoi, 2002).

HURDLES TO PHARMACOGENOMICS

Pharmacogenomics is a new emerging research field that is still in its infancy. Several of the following barriers will have to be overcome before many pharmacogenomics benefits can be realized.

SNPs affecting drug response

The occurrence of single nucleotide polymorphisms (SNPs) in DNA sequence that occur when a single nucleotide (A,T,C,or G) in the genome sequence is altered. SNPs occur every 100 to 300 bases along the 3-billion-base human genome, therefore millions of SNPs must be identified and analyzed to determine their involvement (if any) in drug response. In addition, our limited knowledge of gene linked drug response also complicates the process of drug development. Since many genes are likely to influence responses, obtaining the big picture on the impact of gene

variations is highly time-consuming and complicated.

Limited drug alternatives

Only one or two approved drugs may be available for treatment of a particular condition. If patients have gene variations that prevent them using these drugs, they may be left without any alternatives for treatment.

Pharmaceutical reluctancy

Today, most pharmaceutical companies have been successful with their "one size fits all" approach to drug development. Drug manufacturers may be reluctant due to involvement of huge money to bring a drug to market, to develop alternative drugs that serve only a small portion of the population?

Educating healthcare providers

Multiple pharmacogenomic products prescription by physician to treat the same condition for different population subsets undoubtedly will require more expertise for prescribing and dispensing drugs. Physicians must execute an extra diagnostic step to determine which drug is best suited to each patient. To interpret the diagnostic accurately and recommend the best course of treatment for each patient, all prescribing physicians, regardless of specialty, will need a better understanding of pharmacogenomics.

NEW PHARMACOGENOMIC TECHNOLOGIES

New development in the field of pharmacogenomics has been the result of recent technological advances in high throughput DNA and mRNA analysis and in the processing of these data in an efficient manner. The most dramatic change has been the introduction of techniques for the simultaneous assessment of multiple genes. Initial studies used robotics-based systems tri "print" a series of gene clones onto a silicone-coated glass slide. By labelling the mRNA of interest with a fluorochrome, a correlation was found between the fluorescence intensity emitting from each gene clone and the measured level of gene expression. This approach has been modified to use large gene clones from the Human Genome Project, small oligonucleotides for specific genes, and cDNA derived from differential expression projects (Evans and Relling, 1999). Arrays are currently constructed on nylon filters or glass slides, with slides allowing greater density of genes per experiment and nylon generally being more reproducible. The improvements in robotics and fluid physics are such that up to 64,000 gene clones can be evaluated on a single small 1 inch by 1 inch slide. The gene expression arrays have enabled a degree of genomic analysis which was not feasible earlier. For example, prior to this technology, it is estimated that the quantity of data available from a single array containing 64,000 genes, generated in approximately 48 hours, would have taken a researcher over 20 years to complete by Northern blot analysis (Eisen and Brown, 1999).

Recently, new techniques have emerged to obtain information on patient genotype to increase the throughput of genotype information from genomic DNA in a rapid manner such as fluorescence energy transfer detection, fluorescence polarization (Chen *et al.*, 1999), kinetic PCR (Germer *et al.*,2000), mass spectrometry (Kwok, 1998), oligonucleotides ligation/flow cytometry (Iannon *et al.*, 2000), HPLC fragment analysis (Kuklin, 1997), and mini-sequencing (Syvanen,1999). Analy-

sis of 1,000 to 5,000 genotypes per day is routine in many pharmacogenomics laboratories, with automated multiples assays extending this to 10,000 genotypes per day. The ideal approach for rapid genotying is yet to be defined, but efforts are currently being expanded to test various approaches in the clinical setting. For example, computational biology, or bioinformatics is emerging in a big way to help in the development of pharmacogenomics.

PHARMACOGENOMICS BASED THERAPEUTICS

Rational therapy

The variation in response to drug therapy among patients is potentially regulated by a number of processes including drug transport, drug metabolism, cellular targets and signaling pathways mediated via G-protein-coupled receptors (GPCRs), and cellular response pathways during apoptosis, cell cycle control, etc.During the past 20 years, a large amount of data has been generated that provides a scientific basis for patient-specific selection of medications and their dosage. This includes molecular mechanisms for variation in drug efflux and metabolism, cellular targets e.g. receptor conformation, and heterogeneity in disease pathogenesis and phenotype. Even at a superficial level, the complexity of these different sources of variation is apparent. Heterogeneity in genotype for drug efflux and metabolism, and receptor pathways is not well established for numerous medications which may have additive or even synergistic effects on therapeutic success or toxicity (Evans and Relling, 1999).

Polymorphic drug metabolism

In the context of therapeutics, one of the most developed examples of clinical pharmacogenomics involves the genetic polymorphism of thiopurine methyltransferase (TPMT). TPMT catalyzes the S-methylation of the thiopurine agents such as azathioprine, mercaptopurine, and thioguanine. These agents are commonly used for a diverse range of medical indications, including infants leukemia, rheumatoid arthritis, inflammatory bowel disease, dermatologic disorders, and solid organ transplantation. The principal cytotoxic mechanism of these agents is generally considered to be mediated via the incorporation of thioguanine nucleotides (TGN) into DNA. Thus, thiopurines, the inactive prodrugs, require metabolism to TGN to exert cytotoxicity. This activation is catalysed by multiple enzymes, the first of which is hypoxanthine phosphoribosyl transferase. Alternatively, these agents can be inactivated via oxidation by xanthine oxidase or methylation by TPMT. In hematopoietic tissues, xanthine oxidase is negligible, leaving TPMT as the only inactivation pathways (Mc Leod *et al.*, 2000; Schutz *et al.*, 1993).

SNPs targeted drug

Presence of distinct single nucleotide polymorphisms (SNPs) alters the response in patients with such variations. A SNP is a site on the DNA in which a single base pair varies among individuals in a population. If a SNP is found within a small, unique segment of DNA, it serves as both a physical landmark and as a genetic marker whose transmission can be followed from parent to child. According to theoretical models, if the genotype of a group of individuals with a common disease and a group without the disease are studied, certain genotypes may be consistently associated with those individuals who have the disease. Owing to linkage disequilibrium, alleles of genetic markers in close proximity to a disease-modifying mutation are often found to be associated with the disease, even though they themselves are not involved in disease pathogenesis or drug response. Once localized, these specific chromosomal regions can be analyzed further to identify

disease-associated genes and mutations. This molecular/population genetic approach also provides a strategy to identify genes associated with other phenotypes, such as drug toxicity or therapeutic benefit (Brookes, 1999). For example, the variability in the ?2-adrenoreceptor is the molecular basis for altered response to ?2-agonist. It has recently been revealed by the identification of five distinct single nucleotide polymorphisms, each associated with altered expression, down regulation, or coupling of the ?2-receptor. Alteration at amino acid 16 (Arg>Gly) appears to have relevance in pulmonary disease. Patients homozygous for arginine exhibited a greater response to ?2 agonist medications. For example, the FEV1 response to oral albuterol was 6.5-fold higher in patients with an Arg/Arg genotype at codon 16 compared with Gly/Gly patients at similar plasma drug concentrations (Hon *et al.*, 1999; Relling *et al.*, 1999). In contrast, the alteration at codon 27 (Gln>Glu) has association between the Gln/Gln genotype and an increased incidence of obesity does not appear to influence lung function. The mutant allele for codon 16 (frequency 0.61) and codon 27 (frequency 0.43) have been found to have relatively common characteristics and are, therefore, under intensive investigation for their clinical importance. A less common allele contains a mutation at codon 164 (Thr>Ile) with a mutant allele frequency of 0.05. The clinical significance of this polymorphism was identified in patients with heart failure. A one-year survival was observed in 42% patients with the Thr/Ile genotype compared with 76% patients with Thr/Thr. This finding suggested that patients with the Ile 164 polymorphism and heart failure should be considered as candidates for early aggressive intervention or cardiac transplantation. The ?2 receptor haplotype is more informative than individual SNPs in predicting response to beta agonists in asthmatics (Drysdale *et al.*, 2000; Ligget, 1998; Ligget *et al.*, 2000; Meirheaghe *et al.*, 1999). Variation in SNPs are the most frequently observed in DNA sequence in the human genome, with an estimated frequency of 1 in 1000 bases (Marth *et al.*, 1999).

Comparative genomic hybridization (CGH)

A gene approach is applicable to agents with clearly defined mechanisms of action, metabolism and toxicity. However, the pathways influencing a new agent are often unknown. In such cases, a genome-wide approach with no a priori assumptions for loci of interest may be used. An alternative to low-density genomic approaches such as SNP analysis, is comparative genomic hybridization (CGH) which involves a competitive in situ hybridization of fluorescently labelled test (e.g. tumor) and control (e.g. normal tissue) DNA onto normal metaphase chromosomes from an unrelated healthy donor. A computerized fluorescence microscopy is also used to assess the intensity of signal across each chromosome. The difference in test and control fluorescence intensity reflects the change in DNA amount for specific regions of the human genome. In case of identical chromosomal or chromosomal sub regions are present in both test and control DNA, an equal contribution from each fluorochrome is seen. If certain chromosomal sub-regions are gained or lost in the text DNA, a change in the fluorescent signal is seen. CGH is applicable to DNA only and has primarily been used in the context of tumor biology for identification of novel alterations associated with the acquisition of cancer. With current technology, CGH can detect for gain and loss of genetic material of 5-10 Mb. However, high- level amplification is characterized by gain of as small as 50 kb DNA in regions (Mc Leod and Evans, 2001; Rooney *et al.*, 1999).

Gene Expression

The gene expression has been revolutionized by the development of glass and nylon membrane microarrays techniques. Gene expression is evaluated in all area of medicine, including pharmacology. Initially, the studies have focused on gene expression along biologic pathways and have

provided an increased understanding of the regulation of cellular proliferation and the cell's response to nutrient stimulation (Golub *et al.*, 1999; Ross *et al.*, 2000). The gene expression arrays have been employed in the molecular classification of disease and have highlighted the genetic heterogeneity using diffuse large B-cell lymphomas (DLBCL) with histologically similar appearance. Using a "lymphochip" containing 17,856 genes expressed in lymphoid cells, the presence of two activated B-like DLBCL were demonstrated. More important, the overall survival rate was higher in patients with germinal centre B- like DLCBL than activated B- like DLCBL. Thus, it is proposed to use gene expression arrays as a prospective tool for therapy of individual patients (Alizadeh *et al.*, 2000).

Targeted drug discovery

The potential applications of pharmacogenomics include the identification of novel targets, selective therapy for an individual and to provide tools for predicting efficacy and toxicity prior to clinical use of drugs. Pharmacogenomics also has the potential to make the drug development process efficient by decreasing the number of patients required to show efficacy in early clinical trails (Evan and Relling, 1999).

The SNP projects on human and mouse are focused in an attempt to find specific genes or genomic loci that are associated and expressed during disease under investigation for treatment. These approaches are conducted using gene expression arrays techniques. Disease tissue is used to produce mRNA for comparison with normal tissue. In pharmacogenomics, the SNP and gene expression are vital in the identification of novel targets and subsequently to develop targeted therapy. After identification of the target, the viability of the target in terms of normal and disease tissue expression, pattern of normal tissue expression for toxicity prediction, and frequency of expression in the disease tissue are the major considerations. (Fijal *et al.*, 2000; Mc Leod and Evans, 2001).

PHARMCOGENOMICS IN PUBLIC HEALTH

Pharmacogenomics, initially, is likely to have the greatest benefit for patients in developed countries, owing to expense, availability of technology and the focus of initial research, however, pharmacogenomics will ultimately be useful to world populations. There is clear evidence of ethnic variation in risk and incidence and therapeutic response of diseases. In addition, there are many qualitative and quantitative differences in polymorphic drug metabolizing enzymes among racial groups. For example, the COMT low activity allele is less frequent in African and East Asian population which inactivates methyldopa and one of the most commonly prescribed antihypertensive medications in those regions of the world. In addition, COMT influences the activity of levodopa for Parkinson's disease and the production of estrogen metabolites and thus is associated with breast cancer (Weinshilboum, 1999; Wood, 1998).

CONCLUSION

The one of the major barriers on the progression of pharmcogenomics is the cost involved in individual based genomic sequencing and availability of technologies for the world population with the exception of advanced and well equipped nations. Recently, the technology for gene expression and genotype assessment is only affordable in the research and development setting or in the context of funded research. Thoughtful genomic analysis is needed to justify and direct the further development of pharmacogenomics for rational therapeutics. On the positive side, once the ge-

nome of an individual is correctly sequenced, there will be no need of further repetition for the same person's specific database. Finally, the ethics of genetic analysis is currently under avid discussion and debate, however, country like USA has allotted huge funds for the progress in pharmacogenomic research. It is hoped that pharmacogenomics approach will revolutionize the line of treatment and will be available for all rich or poor persons in every corner of the world.

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ROLE OF BIOSAFETY AND BIOCONTAINMENT FACILITY: CONCERNS AND FUTURE CHALLENGES

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Laboratory Biosafety is the term used to describe the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release in the environment. Microbiological laboratories are special, often unique work environments that may pose identifiable infectious disease risks to persons in or near them. Infections have been contracted in the laboratory throughout the history of microbiology. In response to global concern about emerging and reemerging infectious diseases especially because of the increased national and international transfer of infectious microorganisms and growing concerns about bioterrorism, considerable interest has been generated world-over on the biosafety and biosecurity issues. In India, research work on the various infectious agents has been a regular activity since time immemorial and the realization of the threat from these organisms in spreading to a greater population was always there. However, the preparedness to combat this threat was limited to merely adopting some of the good laboratory practices and decontamination/ disinfection procedures. The use of Biosafety (BS) concept as a scientific method for prevention of spread of infection through man, material and place or cross contamination within the laboratory was limited to a very few Veterinary and Medical laboratories in the country. In addition to the knowledge regarding Biosafety and biocontainment, understanding to laboratory associated infections (LAIs) has also been an evidence of poor practices adopted in handling the infectious agents.

However, with the growing realization of the threat of the exotic, emerging diseases to the country, particularly following the avian influenza outbreak in 2006 widespread understanding has emerged include biosafety as a major administrative and research component in the laboratories that are handling infectious organisms for the safety of man, material and environment (MME). This is evident from the declining trend being shown in the reported LAI cases globally (Fig 1). In India, unfortunately comprehensive data regarding the prevalence of LAIs are singularly lacking. Out of a total of 3947 reports reviewed, only two reports were available from India.

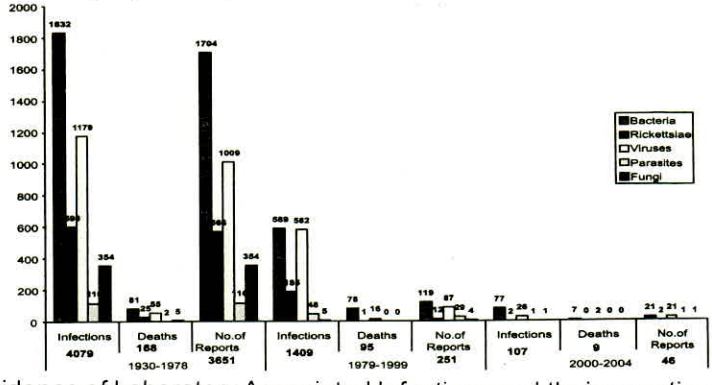

Fig. 1. Global incidence of Laboratory Associated Infections and their reporting status

Biosafety in any microbial research organization is based on certain basic principles of guarded activities which are based on a variety of factors, i.e., the type of infectious material, nature of organism, availability of trained manpower, infrastructure, personal BS devices and administrative requirements. Some of the major principles on which the universal concept of Biosafety works, are discussed below.

Science of Containment: The journey of technological advances from cell to stem cell & nanotechnology, Bunsen burner to laminar flow technology (BSC), from the science of asepsis to clean room environment has led to the evolvement of BS & BC techniques by amalgamation of structural engineering as integral part of biosciences laboratory operations. The containment of biological agents to prevent exposure to laboratory workers and the outside environment is the basic concept of biosafety. Primary containment protects the laboratory workers and the immediate laboratory environment from exposure to biological agents, which is achieved through good microbiological technique and the use of safety equipment and personal protective equipment. Secondary containment protects the environment outside the laboratory, and is provided by facility design and operational procedures. Facility design and security features include physical separation of laboratories from public access, specially designed ventilation systems (to prevent airborne biological agents from migrating outside the laboratory), and autoclaves. These design features protect personnel working outside the immediate laboratory, as well the outside environment. The World Organization of animal Health (OIE) and WHO containment guidelines are the general guiding rules to be followed globally. Besides these, a number of other countries and their organization have formulated their own guidelines. Forerunners of such organizations include; the American Biosafety Association (ABSA), European Biosafety Association (EBSA), Canadian Biosafety Association (CBA), British Biosafety Association (BBA) and Asia Pacific Biosafety Association (APBSA).

Good Laboratory Practices: The use of good microbiological technique is the most important element of containment. Personnel working with biological agents must be aware of hazards, and must be trained to safely handle and dispose of these materials. Each laboratory should prepare a laboratory biosafety manual to provide general policies and procedures when working with biological agents. The Biosafety manual is a comprehensive document that specifies all the procedures involved in handling, processing, transport, decontamination of the men, material and place and disposal of all the infectious material being handled in the laboratory. As far as possible these procedures should be prepared in the form of Standard Operating Protocols (S.O.P.) and should be displayed at all the appropriate places so that these are accessible to all the workers in the laboratory, right from the cleaning staff to the researcher. SOPs may be generated covering overall laboratory activities or may be highly specific to handle a pathogen of high risk and public health concern. A variety of workplace or country specific SOPs have been developed so far that are commonly referred for use or modifications. Guidelines developed by Center for Disease Control, Atlanta, WHO, ABSA are considered of international standards of Biosafety and these guidelines can be used to develop country specific SOPs and guidelines to fulfill the required Sanitary and Phytosanitary (SPS) measures.

Biological Hazard Information: Laboratory workers must be knowledgeable of the hazards associated with the biological agents present in the laboratory, and have hazard information available to them. Signs must be posted at or on the access doors indicating that biological agents are used within the room. The sign must include the universal biohazard symbol, the name of the agent(s)

present, any specific entry requirements (such as personal protective equipment or immunization), and the name and telephone number of the individual working on the organism and/or other responsible person(s).

Storage and Security of High Risk Pathogens: Global events in the recent past have highlighted the need to protect laboratories and the materials they contain from being intentionally compromised in ways that may harm people, livestock, agriculture or the environment. Laboratory biosecurity measures should be based on a comprehensive programme of accountability for pathogens and toxins that includes an updated inventory with storage location, identification of personnel with access, description of use, documentation of internal and external transfers within and between facilities, and any inactivation and/or disposal of the materials. Every principal investigator in the laboratory has the responsibility of ensuring that his or her laboratory implements sufficient security measures and procedures to prevent unauthorized access to biological agents. Select Agents and other higher risk microorganisms and toxins must be stored in a locked container, and the PI must maintain an inventory with sufficient detail to enable identification of missing materials.

Biological agents must be stored using double containment. Both the primary and secondary containers must be durable and leak proof so as to prevent accidental exposure. Primary containers must be clearly labeled as to the identity of the agent and should include the universal biohazard symbol as physical space on the container permits. At a minimum, secondary (or outside) containers must include the universal biohazard symbol. Freezers, refrigerators, and other storage areas must also be labeled with the biohazard symbol; exceptions to this policy will be considered on an individual basis by the BSO. Waste, and contaminated equipment or other objects to be decontaminated must also be labeled with the Biohazard Symbol.

Safety training: All the staff members working in the containment laboratory needs to undergo two types of training programmes: 1. the Primary training before he starts his work in the laboratory and 2. Intermittent refresher training so that they are updated on the changing Biosafety concepts. The training programme, in general should cover areas such as general biosafety work procedures, biohazardus waste disposal, emergency response, etc. Laboratory biosecurity training, distinct from laboratory biosafety training, should be provided to all personnel. Such training should help personnel understand the need for protection of such materials and the rationale for the specific biosecurity measures, and should include a review of relevant national standards and institution specific procedures.

Safety Equipment: A. Safety equipment is most effective at minimizing exposure when workers are trained on the proper use of such equipment, and the equipment is regularly inspected, maintained and calibrated A biological Safety Cabinet (BSC), besides safety centrifuge cups and other engineered controls are also used to minimize exposure to biological agents. Biological safety cabinets (BSCs) are the most important safety equipment for protection of personnel and the laboratory environment, and most BSCs also provide product protection. Three types of biological safety cabinets (Class I, II, III) used in microbiological laboratories Open-fronted Class I and Class II biological safety cabinets are primary barriers which offer significant levels of protection to laboratory Personnel and to the environment when used with good microbiological techniques. The Class II biological safety cabinet also provides protection from external contamination of the materials (e.g., cell cultures, microbiological stocks) being manipulated inside the cabinet. The gastight Class III biological safety cabinet provides the highest attainable level of protection to person-

nel and the environment.

Table:1 Comparison of Biosafety Cabinets

Type	Face velocity (1fpm)	Airflow Pattern	Radionuclides/ Toxic Chemicals	Biosafety Level(s)	Product Protection
Class I* open front	75	In at front; rear and top through HEPA filter	No	2,3	No
Class II Type A	75	70% recirculated through HEPA; exhaust through HEPA	No	2,3	Yes
Type B1	100	30% recirculated through HEPA; exhaust via HEPA and hard ducted	Yes (Low levels/volatility)	2,3	Yes
Type B2	100	No recirculation; total exhaust via HEPA and hard ducted	Yes	2,3	Yes
Type B3	100	Same as IIA, but plena under negative pressure to room and exhaust air is ducted	Yes	2,3	Yes
Class III	NA	Supply air inlets and exhaust through 2 HEPA filter	Yes	3,4	Yes

B. Personal Protective Equipment (PPE): Safety equipment also includes items for personal protection. Personal protective equipment is often used in combination with BSCs and other devices that contain the agents, animals, or materials being handled. Personal protective equipment includes gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. These PPE are used to supplement the containment provided by laboratory practices and safety equipment. In some situations in which it is impractical to work in biological safety cabinets, personal protective equipment may form the primary barrier between personnel and the infectious materials. Examples include certain animal studies, animal necropsy, agent production activities, and activities relating to maintenance, service, or support of the laboratory facility.

Risk assessment: The backbone of the practice of biosafety is risk assessment. While there are many tools available to assist in the assessment of risk for a given procedure or experiment, the most important component is professional judgment. Risk assessments should be performed by the individuals most familiar with the specific characteristics of the organisms being considered for use, the equipment and procedures to be employed, animal models that may be used, and the containment equipment and facilities available.

Laboratory facilities are designated as basic - Biosafety Level 1, basic - Biosafety Level 2, containment - Biosafety Level 3, and maximum containment - Biosafety Level 4. Biosafety level designations are based on a composite of the design features, construction, containment facilities, equipment, practices and operational procedures required for working with agents from the various risk groups (Tables 2 and 3). In India, such a classification is non-existent for the Indian context. Efforts are required to form a committee of experts from the veterinary and medical fields to review the pathogens present in the country recommend the risk groups under Indian context. Countries (regions) should draw up a national (regional) classification of microorganisms by risk group, taking into account the pathogenicity of the organism being handled, the mode of transmission and host range of the organism, the local availability of effective preventive and treatment mea-

sures. The assignment of an agent to a biosafety level for laboratory work must be based on a risk assessment. Such an assessment will take the risk group as well as other factors into consideration in establishing the appropriate biosafety level.

Construction, commissioning, operation and maintenance of Biosafety laboratories

There has been a growing realization on urgent basis to prevent potential hazard for the Laboratory Associated Infections to the laboratory workers and the environment. Consequently, a large number of laboratories having various levels of Biosafety are being set up both for human and animal health. Following the diagnosis of Highly Pathogenic Avian Influenza at HSADL in February 2006 and July, 2007, there has been a growing realization amongst the public health experts from veterinary as well as human fields regarding the need of carrying out work related to the zoonotic pathogens under various levels of containment to prevent the accidental spread of infection in the environment and among the laboratory personnel. As a consequence at least a dozen laboratories with BSL2 plus facilities are coming up all over the country(Table 3). Setting up of these laboratories and commissioning them for their optimum utilization will reduce the environmental contamination and provide a safer laboratory environment to the worker.

Table 2: Existing, upcoming and proposed Biosafety laboratories in the country

	Laboratory	Organization	BSL status
Existing	HSADL, IVRI, Bhopal	ICAR	BSL-IV
	NIV, Pune	ICMR	BSL-III
Upcoming	NIV, Pune	ICMR	BSL-IV
Proposed	CCMB, Hyderabad	CSIR	BSL-IV

Our decade long experience in operating and maintaining a BSL - IV facility has thrown up several challenges and has made us realize that the Biosafety as a concept is very dynamic and no single laboratory in the world can claim itself to have a foolproof and a stable biosafety model. Moreover, the biosafety requirement of individual laboratory depends upon a large number of factors, viz., the kind of pathogen/infectious agent being handled, the number of researchers working inside the containment complex, the monetary and infrastructure resources available to meet the recurring costs (which is several times higher than a conventional open laboratory) and most importantly, the availability of trained biosafety and engineering manpower to supervise the biosafety procedures. The laboratory biosafety protocols, engineering and design is primarily based on these factors. It is therefore a great challenge in front of the planners and implementers to make these laboratories sustainable and effective.

1. Biosafety Laboratory construction and commissioning: The construction and commissioning of a Biosafety laboratory requires a systematic review and documentation process signifying that specified laboratory structural components, systems and/or system components have been installed, inspected, functionally tested and verified to meet national or international standards, as appropriate. The respective building system's design criteria and design function establish these requirements. In other words, laboratories designated as Biosafety Levels 1-4 will have different and increasingly complex commissioning requirements. Geographical and climatic conditions, such as geological fault lines or extreme heat, cold or humidity may also affect the laboratory design and therefore the commissioning requirements. Upon the completion of the commissioning process, the pertinent structural components and support systems should be subjected to the

various operating conditions and failure modes that can be reasonably expected, and will have been approved.

2. Availability of financial resources on a sustainable basis: The construction of Biosafety laboratory requires a large capital and also involves making provision for sufficient capital to meet the routine operation costs, maintenance and for the periodic need based modifications depending on the pathogens being handled in the containment facility. In a large number of cases, it has been observed that the finances required for the construction of a laboratory are available as a one-time plan grant but the budget required for maintenance and operation of these laboratories is woefully inadequate. Whereas, such shortcomings can be managed in open laboratories, this issue can itself pose a serious health hazard as the failure of maintenance of containment facility could result in accidental release of the pathogens in the environment.

3. Availability of Biosafety specialists: The risk of handling infectious pathogens to the worker, environment and the general population has always been recognized and precautions at individual level were always taken to mitigate this risk. The need to tackle this issue through a documented scientific approach has been recognized only in recent years. This has given rise to the requirement of Biosafety specialists in large numbers and our country is woefully short of these specialists. Biosafety as a science is meaningful and effective only when the engineering component of biosafety amalgamates with the traditional fields of microbiology, biotechnology and other areas of life sciences. Such an unique combination of specialists can only be obtained through the evolvement of comprehensive training programmes to cater to these requirements. Such training programmes can be evolved in line with the training courses developed in bioinformatics where the training involves the knowledge in the field of biotechnology, life sciences and the computer sciences. The universities should seriously consider developing postgraduate degree or diploma courses in Biosafety. This could go a long way in meeting the shortage of Biosafety experts in the country and could also open up new job opportunities for the young generation involved in the field of biosciences and areas of engineering.

In conclusion, biosafety in our laboratories are the need of the day, however, actual implementation of the biosafety concepts in its letter and spirit is a Herculean task. It requires a cohesive action from the planners; researchers, medical, veterinary and pure science professionals and the private entrepreneurs involved in the pharmaceutical and biologicals industries. One must realize that each and every organism that an individual is handling is a potential biohazard and he has to own up the responsibility of ensuring that this is not being exposed to the environment and population. This is the only way to ensure safe environment and healthy population.

Table 2: Requirements for different levels of biosafety laboratories as per HSADL experience adopted from various sources*

		Biosafety Level		
	Laboratory Requirement	2	3	4
A.	Laboratory Setting and Structure			
1.	Not next to a known free hazard	Y	Y	Y
2.	Workplace separated from other activities	Y	Y	Y
3.	Personnel access limited	Y	Y	Y
4.	Protection against entry/exit of rodents & insects	Y	Y	Y
5.	Sterilization of liquid effluent	-	Yes &	Yes &

			Monitored	Monitored
6.	Isolation by air lock, continuous internal air flow	-	Y	Y
7.	Double door entry	-	Y	Y
8.	Input and extract air to be filtered using HEPA or equivalent	-	Ya	Yb
9.	Mechanical air supply with fail safe system	-	Y	Y
10.	Laboratory sealable to permit fumigation	-	Y	Y
11.	Incinerator for disposal of carcass and waste	-	Y	Y on site
B. Laboratory Facility				
12.	Class 1/2/3 exhaust protective Biosafety cabinets available	Y	Y	Y
13.	Direct access to autoclave	Y	Y, with double door	Y, with double door
14.	Specified pathogen stored in lab	Y	Y	Y
15.	Double ended dunk tank required	Y	Y	Y
16.	Protective clothing not worn outside the laboratory	-	Preferable	Y
17.	Showering required before exiting laboratory*	-	-	Y
18.	Safety officer responsible for containment	Y	Y	Y
19.	Special training for the staff in the requirements needed	Y	Y	Y
20.	Radioisotope handling and storage as per BARC specifications	Y	Y	Y
C.	Laboratory discipline			
21.	Warning notices for containment area to be displayed	Y	Y	Y
22.	No eating, drinking and smoking inside the lab	Y	Y	Y
23.	No storage of food in laboratory except specified locations	Y	Y	Y
24.	Laboratory must be lockable	Y	Y	Y
25.	Authorized entry system for personnel	Y	Y	Y
26.	On entry, all clothing removed & clean laboratory clothes put	-	Y	Y
27	On exit, all laboratory clothing removed, individual must wash and then shift to clean area	-	Y	Y
28.	Individual must shower prior to shifting to clean area	-	-	Y
29.	All accidents reported	Y	Y	Y
30.	Use of personal protective equipment (PPE)	Y	Y	Y
D.	Handling specimens in the laboratory			
31.	Packaging requirements to be advised prior to sample submission	Y	Y	Y
32.	Incoming packages opened only be the trained staff	Y	Y	Y
33.	Movement of pathogens from an approved laboratory to another restricted.	Y	Y	Y
34	Standard Operating Protocols (S.O.P.s) covering all areas	Y	Y	Y

[a]single on input &extract bsingle on input and double at extract * WHO, OIE, CDC.

SESSION VII

IPR and Commercialization Issues for Biologicals

Chairman : **Dr. L.M.S. Palni**

Co-Chairman : **Dr. H.S. Chawla**

Rapporteur : **Dr. Thanislass**

LEAD PAPERS

VII.1 IPR Issues Related to Research & Development of Immuno-Biotechnology Based Biologicals
H.S. Chawla

VII.2 Product Profiling of Veterinary Biologicals
K. S. Palaniswami

VII.3 Bio-safety and ethical issues related to production of genetically engineered biologicals
Gaya Prasad Minakshi

VII.4 Cell Culture Scale up Techniques in Biological Production: An Overview
R K Singh

VII.5 Recent Trends in the Development of Veterinary Vaccines
S.K. Garg and Sameer Shrivastava

ABSTRACTS

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Bhaskar Ganguly, Sudhir Kumar, Umapathi V., Tanuj Ambwani

7.02 Biotechnology in Indian pharmaceutical industry
S.P. Singh, Prof. and Sanjay Sharma

7.03 Evaluation of Immunopotentiating effects of cow urine on captan induced Immunotoxicity in chicken lymphocytes
Sonu Ambwani, Tanuj Ambwani, D.K.Agrawal and R.S.Chauhan

7.04 Challenges of commercialization of therapeutic recombinant antibodies for India
Jagveer Rawat

7.05 Immunopotentiating effects of cow urine on allethrin induced Immunosuppression in chicken lymphocytes cell culture system
Sonu Ambwani, Tanuj Ambwani, G.K. Singh and R.S.Chauhan

IPR ISSUES RELATED TO RESEARCH & DEVELOPMENT OF IMMUNO-BIOTECHNOLOGY BASED BIOLOGICALS

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Intellectual property right (IPR) protection plays a key role in getting an advantageous position in the competitive world for achieving economic growth. This has become more pronounced in the globalized economy with the obligations in the field of IPR in the WTO regime. With the globalization of trade and commerce, it has become important to enrich our knowledge about IPRs. IPRs are important not only because India as a member is required to accede to the conditions of an international agreement but also because they offer possible mechanisms for stimulating research, enabling access to technology and promoting enterprise growth with an ultimate aim to benefit human population. The importance of IPRs can be gauged from the fact that most globally competitive corporations strategically protect their intellectual properties in all potential markets/countries by filing patents and other IP arrangements. Although each country implements intellectual property laws at the national level, the TRIPS (Trade Related Aspects of Intellectual Property Rights) Agreement imposes minimum standards on patents and other IPs. As a result of the commitments made under the TRIPS Agreement, India has enacted and amended their existing norms of IP protection to be TRIPS compliant for which the deadline was December 2004.

IPRs can be defined as the rights given to people over the creation of their minds. They usually give the creator an exclusive right over the use of his/her creation for a certain period of time. Intellectual property includes patents, copyrights, trademarks, geographical indications, industrial designs, integrated circuits and trade secrets. The protection of IPRs is binding and legally enforceable.

Patents

A patent is a government granted exclusive right to an inventor for the development of a new product or process involving an inventive step, which is capable of industrial application. This will prevent others from practicing i.e. making, using or selling the invention. A patent is a personal property, which can be licensed or sold like any other property. The purpose of a patent is to encourage and develop new innovations. The Patent Law recognizes the exclusive right of a patentee to gain commercial advantage out of his invention. There are three criteria of novelty, inventiveness and usefulness to issue a patent for the innovation. In the patent adequate disclosure should be made so that others can also work on it. It should have the features: i) be a written description; ii) enables other persons to follow; iii) adequate and iv) deposit mechanism. The present law, Patents Act 1970, amendment 2005 is effective from January 1, 2005. Process and product patents on all items including food, agro-chemical and pharmaceuticals have been allowed making The Patents Act fully TRIPS compliant.

The patent system was developed as a means to reward inventions, which would be useful to the society. However, in order to ensure the interests of society, as per the Indian Patents Act, certain things have been excluded from the purview of patentability. The sections relevant to the title under consideration which are excluded from patentability are:

Section 3(d): the mere discovery of any new property or mere new use for a known substance or the mere use of a known process, machine or apparatus unless such known process results in a new product or employ at least one new reactant;

Section 3(e): a substance obtained by merely admixture resulting only in the aggregation of the properties of the components thereof or a process for producing such substance:

Section 3(i): any process for medicinal, surgical, curative, prophylactic (diagnostic therapeutic) or other treatment of human beings or any process for a similar treatment of animals to render them free of disease or to increase their economic value or that of their products:

Section 3(j): plants and animals in whole or any part thereof other than microorganisms but including seeds, varieties and species and essentially biological processes for production or propagation of plants and animals:

Section 3(p): an invention which in effect, is traditional knowledge or which is an aggregation or duplication of known properties of traditionally known component or components:

The first patent on living organism was granted to Dr Chakrabarty in 1980 for a new micro-organism *Pseudomonas* which had four plasmids and therefore more useful in dispersing oil slicks than the natural organism containing only one such plasmid. US Supreme Court decided that microorganism should not be precluded from patentability for the objection raised by USPTO on the basis of "product of nature". This precedent is being followed even today to define the patentability of microorganisms.

Microorganisms *per se* can be claimed for protection provided they are not mere discovery of organisms. It is mandatory to deposit the biological material in International Depositary Authority (IDA). In India, Institute of Microbial Technology (IMTECH), Chandigarh is a recognized IDA for some category of microorganisms. If an applicant mentions a biological material in the patent specification then disclosure requirements prescribed for biological materials have been notified in the list of the Central Government or for indicating its source and geographical origin [Section: 10,4(d)].

Animal patents: The question of whether multicellular animals could be patented was examined by the USPTO in 1980s. In 1987, Ex Parte Allen case the key issue was the patentability of polyploid pacific coast oysters that had an extra set of chromosomes. However, USPTO rejected the patent application on the ground of obviousness. On April 12, 1988 USPTO issued the first patent on transgenic non-human animal "Harvard Mouse" (U.S. Patent No. 4,736,866) developed by Philip Leder (Harvard University) and Timothy Stewart. After initial reluctance by the EPO, European patent was issued in 1992. (Chawla, 2005). The new provisions of EPC in 1999, Rule 23c states that inventions concerning biological materials, such as DNA, microbiological process. plants, and animals are patentable only if "the technical feasibility of the invention is not confined to a particular plant or animal variety". Further the EPC has prohibited patents on plants and animals as per EPC Article 53(b) mentioned in the category of plants and on *ordre public* or morality [Article 53(a)]. EPC has stated that certain inventions are excluded from patentability whose exploitation is contrary to *ordre public* or morality, namely: processes for cloning human beings; processes for modifying the germ line genetic identity of human beings; use of human embryos for industrial or commercial purposes; and processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or

animal, and also animals resulting from such processes.

Modified animals are patentable in USA, Japan, Korea, Hungary, South Africa and few other countries but not in India. Like wise Patent offices of USA, Japan and Australia grant patents on human body parts such as limbs, organs and tissues. The making of human body parts is not viewed as invention since they exist in nature but modified or isolated body parts are viewed as multi-cellular organisms and treated as such for patentability if they meet the statutory requirements.

Cloning: Cloning is the process of transferring nucleus of an adult multicellular organism's cell to an unfertilized egg of the same species while transgenic cloning is when a particular gene is added to the nucleus of an adult organism cell before its transfer to an unfertilized egg of the same species. Dolly the first mammal sheep was created in 1997 by cloning. Creation of animals by cloning is patentable in some countries. However, patenting of human cloning issue varies in different countries. Japan banned human cloning in 2001 but permitted researchers to use human embryos that were not produced by cloning. Recently in July 2004 Japan Government Science Council has permitted limited cloning of human embryos for scientific research. Britain and South Korea also allow cloning of human embryos for therapeutic purposes. However, United States prohibits any kind of human embryo cloning but allows patenting of animal cloning. In the controversial issue of cloning, no attempt has been made to implement strict legislation in US, but in Europe in July 1998, a European directive (98/44/EC) was adopted on the legal protection of biotechnology inventions. Another major difference is that US patents on the human embryonic stem cells have been granted while in Europe the ethics of stem cells patentability is still a controversial subject of debate

Biological compounds: Biological compounds, such as DNA, RNA and proteins, are not themselves living, but naturally occur in nature. The ability to isolate genes and produce the proteins they encode has enormous commercial impact. The availability and scope of patent protection on genes and genome-related technologies is considered vital for the survival and success of the biotechnology industry. Under U.S. Patent law, DNA sequences are considered chemical compounds by the USPTO and are patentable as compositions of matter. In its "Utility Examination Guidelines," the USPTO explained that isolated and purified DNA molecule that has the same sequence as a naturally occurring gene is different from the naturally occurring compound as it is processed through purifying steps that separate the gene from other molecules naturally associated with it and hence eligible for patent protection. If a patent application discloses only nucleic acid molecular structure for a newly discovered gene, and no utility for the claimed isolated gene, the claimed invention is not patentable. Since one of the requirements of a patent is utility. Indian Patents Act, 1970 allows inventions on isolation for a substance like DNA, gene sequences are patentable if function has been ascribed to that gene sequence. The Japanese Patent Office also points out that since "the aim of the patent law is to develop industries, only inventions that are useful or having industrial applicability are patentable".

When to file patent: It is a common experience that through ignorance of patent law, inventors act indiscreetly and jeopardize the chance of obtaining patents for their inventions. The most common of these indiscretions are: a) to publish their inventions in scientific and technical journals or newspapers, before applying for patent; b) to wait until their inventions are fully developed for commercial working, before applying for patents. Delay in making application for a patent involves risks, namely, i) other inventors might forestall the first inventor in applying for the patent, and ii) there might be either an inadvertent publication of the invention by the inventor himself or the

publication thereof by others independently of him.

The purpose of a patent is to promote the progress of science and useful arts. The patent law promotes this progress by giving the inventor the right of exclusion. In exchange for this right to exclude others, the inventor must disclose all details describing the invention, so that when the patent period expires, the public may have the opportunity to develop and profit from the use of invention. A patent is enforced in the country, which issues it, meaning thereby territorial in nature. For each country, a separate application is to be filed in that country where protection is sought. However, India is a member of Patent Cooperation Treaty (PCT), a multilateral treaty which entered into force in 1978. Through PCT, an inventor of member country (contracting state) of PCT can simultaneously seek patent protection for his/her invention in all/any of the member countries, without having to file a separate application in the countries of interest, by designating them in the international PCT application. All the patent offices in India have been designated as receiving offices (RO) for acceptance of PCT applications. This PCT route helps in saving the initial investments towards filing fees, translation, etc. World Intellectual Property Organization (WIPO) office situated at Geneva coordinates all the activities of PCT. Every application shall be made in the prescribed forms.

Patenting and Pharma industry: The large volume of production capacities is one of the basic advantages of the Indian Pharmaceutical Industry (IPI) contributed by the big and small producers. It has resulted in close competition and relatively low prices of the drugs as compared to other countries, due mainly to The Patent Act of 1970. Though a sizeable percentage is engaged in the production of formulations, yet production capabilities in research-intensive bulk drugs are also increasing. Importantly, besides producing drugs that are off patent, the domestic industry has also developed expertise in process capabilities to produce drugs that are still on patents. The most successful strategy so far adopted by the IPI is to develop the indigenous version around the original product, which becomes a close substitute to the patented product. Once a close substitute to the patented product arrives in the market, many producers follow the suit resulting in reduction in the price, which happened in the case of Amoxycilin and Ranitidine. Bit it should be admitted that the process patents which enabled the Indian firms to produce a 'new' product without much investment in R and D has brought in a sense of complacency about their performance among the pharmaceutical firms. Due to poor linkages between research laboratories and industry, utilization of such research and research infrastructure facilities have remained at low level. Obviously, the per capita R and D expenditure suggests that much of the investment is perhaps going towards reverse engineering rather than towards new product development. This low investment in R and D is reflected in the lower number of patents filed by and granted for the residents compared to the non-residents in India.

It is observed from the filing of the Patent Application in India that foreign national or companies file 75% applications. But in Japan, the picture is just reverse where the national companies or individuals file the 80% applications. In Europe and USA, the picture is 50% foreign applicants and 50% national applicants. So, it is clearly understood that the monopolistic right exist in the hand of foreign companies particularly, for the new product and new process in the emerging field of technology. This is a great issue for the pharmaceutical industry in India beyond 2005.

India's key strength is in incremental inventions especially in pharmaceutical formulation and new drug delivery system. Recent debates started after the explanation given in the provision of non

patentable invention under section 3(d) whether only new chemical entity should be patentable or formulation can be considered as a subject matter for patent or even include diverse structural forms of molecules or polymorphs or derivatives or different salts. In that respect it can be concluded that the subject matter is to be judged not only by examining mere definition of the Act but also by the effect of the said different forms. In the recent amendment of the Act different safeguard provisions like Compulsory license, rights of patentee, certain acts not be considered as an infringement are provided to address the health issues and to protect the interest of Indian generic industry.

It is suggested to share the benefit of patent system by using the disclosure of technology open for utilization without infringement and in some cases going to license agreement with the patent holder by transferring technology. It is advised to the leaders of the pharmaceutical companies to create a good network among academia, industries & R&D institutes to use the available knowledge for the development of the product because it is not feasible to bring all kinds of knowledge under a single umbrella for creating a commercial work.

Present Status of IPR Management

Presently the aims of publicly funded institutions such as universities, colleges, autonomous bodies and public sector undertakings are multifaceted which are not purely driven by economic considerations but driven by considerations of social obligations, political objectives and will of a nation. This approach has helped in creating a pool of highly educated population and also building an inherent strength in research and development in agriculture related technologies as well as in basic industries. However, this system has bred complacency, which blunts the spirit of innovation and fire for being ahead of others.

Globalization has taught us many new lessons by opening our eyes to the existing and forthcoming ground realities, which cannot be shunned away just because we do not happen to like them. These realities are going to stay. The likely impacts of globalization started becoming a part of our age old thought process and life style when we decided to become members of the World Trade Organization. Managing creativity within the innovation process is not easy. From providing initial impetus for new ideas and a means of collating and evaluating them to determine the most appropriate exploitation strategy and selecting delivery partners, innovation is a process and can therefore be managed (Chawla, 2007).

Management of IPRs requires capacity building as per their needs. Capacity building is never monolithic in nature but a multidimensional and complex activity. Capacity building should be in all the areas viz. IPR management, information and documentation, patent search and analysis, techno-legal drafting of patent applications, patent litigation, licensing, valuation and negotiating IP licensing deals. No exercise at a national level can succeed if all or most players from innovators to entrepreneurs, scientists to students, NGOs to farmers are not engaged in the activity. The Development of skills and competence to manage IPRs and to leverage its influence should be given a major thrust. This area calls for significant technological insights and legal expertise and should be handled differently from the present, and with high priority.

Thus in the present day scenario one must understand the importance of IPRs and should protect it in proper way before disclosing to the whole world. In case of invention one should protect it by patenting and then publish it.

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PRODUCT PROFILING OF VETERINARY BIOLOGICALS

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While the technologies of genetic intervention is nearly two decades old, the knowledge of DNA structure dates back over five decades. The technology explosion is for isolating, amplifying, sequencing, reading and inserting DNA, with scaled up and automated methods. When the system of automation is getting disseminated, more number of researchers' uses these techniques with ease and precision, resulting in availability of hundreds of products with wealth of data.

Coupled with developments of information management, this wealth of data lead to prediction of genetic structure and function increasing the knowledge on products. It will always be impossible to evaluate the full potential, both positive and negative of such knowledge unless otherwise the products are profiled and bio listed.

The impact of gene-based technologies in livestock production is growing. Genetic modification of animal is now possible, though it has adverse physiological disturbances. Administration of gene-based growth hormones to improve the production has drawn routine attention or cautious approval/rejection.

Control of diseases in animals using GM technologies is a much less contentious issue. Genetically modified vaccines with specificity and marker potential are in use. DNA typing is helping with rapid and precise diagnosis. Molecular epidemiology is the current trend in control of trans boundary diseases like avian flu and new types of FMD viruses.

Genetic improvement of animals for disease resistance or production traits by using marker technologies and functional genomics to target DNA sequences are two parallel tracks. Production of therapeutic proteins and compatible tissues for transplants are two areas of genetic modification in human health.

While ethics and public acceptability are two areas of concern in the entire gene based technologies, immunological and biotechnological products in animal health are less contentious issues.

The profiling of such products reduce cost and increase the consistency by promoting technology sustenance and improvement, debate, team research and containment of overlapping research.

Product profiling –What is to be done?

I. Application of gene based technologies inanimal sciences

Animal Feeds

- Intervention in corn and soybean cultivation without herbicide and insecticide to reduce cost of cultivation and as a concept in organic farming.
- Intervention to reduce P and N_2 excretion and provide the valuable amino acid balance

Animal Production

- Genetic modification
- Disease resistance
- Production traits
- Insertion of growth hormone DNA in fish
- GM animals
- Therapeutic protein in milk
- Compatible tissues for transplant

Animal Health

- Antibiotic and drug use – restriction –alternate protection methods.
- A cost saving method and towards organic farming
- Conventional novel vaccines and GM vaccines
- DNA typing and diagnostic kits with other principles
- DNA techniques in molecular epidemiology
- Interaction of pathogens with genotype of animals (e.g. scrapie)

What is the current status of the products developed?

- Product for the project purpose-forget!!
- Product for degree alone-no follow up action in uncovered areas
- Generated Technologies un-utilized
- Lack of university or institute repository
- Revival problem and culture loss
- Repeatability and reproducibility
- Inconsistent supply of reagents for want of resources
- Fear of adverse comments

II. How to profile veterinary diagnositcs and vaccines?

Name the product first

- A novel word to remember
- Direct or indirect indicator of disease and purpose
- Field application example : dipsticks
- Institution of development

Give a product overview

- Protection purpose-strain specific?
- Diagnostic /epidemiological kit
- Combined products or not

- Manufacturing technique
- Bio-partners involved
- Funding agency and period
- Is Outcome Consistent with national program?

Overview of diseases to which the product was developed

- Classification under OIE
- Diagnostic complexity in differentiation
- Duration of immunity Description
- Epidemiology / Transmission

Application of the product

- Direct by farmers
- Laboratory technicians on receipt of sample
- Veterinary services providers
- Field veterinarians
- Expert field diagnosticians
- Para - professionals

Product seed or base technology- a short description

- Source of seed
- History of such seed
- Seed modification
- Technology Base
- Improvement or modification
- Scope for further improvement

Manufacturing technique of the diagnostics or vaccine

- Modified live or attenuated vaccine
- Antigen mass-capsular/ fimbrial / subunit / multi component
- Inactivation technique and period
- Adjuvant used to potentiate response
- Recombined product / genetically modified
- Antigen or Antibody specificity and source in diagnostics
- Conjugates in ELISA kits / Chemiluminescence techniques

Product development and clinical trial support

- Technology in development

- Internal validation as per OIE standards
- External validation
- National laboratories
- Referral laboratories
- Field trial under different agro climatic conditions
- Feed back on performance
- Diagnostic correlations
- Disease control on outbreak
- Duration of immunity
- Maintenance of seed material and periodical refreshing

Storage and expiry information

- Storage life in ambient temperature/field
- Under refrigerated condition [4°C]
- Under -20° C
- Under lab conditions [-70°C / -160°C]
- Manufacturing date and batch no

Therapy areas or indications

- Diseases to be protected
- Age at administration
- Booster if need be
- Collection of sample for diagnostic / sero - conversion tests
- Expected period of protection

Dosage and administration

- Vaccines- Reconstitution or directions for administration and disposal
- Diagnostic Products- method of use and disposal

Caution

- Practical concerns
    - Veterinary public health
    - Bio – security
    - Administration in advanced pregnancy.
- Method of bio-waste disposal
- Bio – safety issues
- Half – life in case of radioisotopes

Veterinary License

- If applied- name of the agency
- Information if license obtained
- Name of the regulatory bodies - national and international
- Institutional Bio-safety committee / RCGM approval

IPR Issues

- Patents are to protect inventions
- Code number for the 'process 'or 'product'
- Information if applied to granting authority
- Clarity on living/natural product-divergent opinion
- Current situation is open to interpretation
- Global Harmonization of patent standards necessary

Publications / Presentations

- Comments received on discussion/referees
- Citations
- Reviews
- Handouts distribution

Incorporation In Online Catalogue

- University or Institute website and their links.
- National listings in professional societies
- International affiliated institutions
- Listings in accessible websites

Funding Agencies

- Project title
- Project cost
- Year of completion
- Copy of the final report on net

Private Public Partnership

- Helpful in validation
- Helpful in commercialization
- Product scaling up by industry
- Market study and distribution through established network
- Shared inputs

Commercialization

- Permission from funding agency
- Product listing in NRDC
- Market network and Availability
- Import Substitution
- Quality Assurance by developers- every 5 batches
- Price stability

Contact Person

- Name /Designation/institution
- Further clarification or interaction
- Royalty issues if any

Where to submit the profile?

The information furnished above may be submitted to National Research and Development Organizations, all vaccine manufacturers, other SA/VUs, central institutions, funding agencies, professional societies etc. through their research administrator.

These are the broad format and the same may be abridged wherever necessary.

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Anon (2003), Book of Extended synopses, FAO/IAEA International Symposium on Application of Gene based Technologies for improving Animal Production and Health in developing countries, IAEA-CN-110, Vienna, Austria.

BIO-SAFETY AND ETHICAL ISSUES RELATED TO PRODUCTION OF GENETICALLY ENGINEERED BIOLOGICALS

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Introduction

In the past three decades several new technologies including recombinant DNA have been developed to produce genetically engineered biologicals including vaccines, diagnostics, bioactive proteins, biocides and microbes for medical, veterinary and other applications. These developments have created concerns for safety and quality of such products. Development of an effective regulatory system for genetically engineered biologicals has been the subject of increasing discussion among researchers, industry and policy developers, as well as the public. Since recombinant DNA techniques are relatively new scientific techniques, there is limited information about recombinant products and organisms. The issues associated with the regulation and biosafety of genetically engineered products pertain to environmental impact, human food safety, animal health and welfare, trade and ethics. To regulate this new and powerful technology predicated on limited background information is a challenge not only for the regulators, but also for the developers of such products, who strive to prove that the recombinant biologicals are safe and merit bio-equivalency to their conventional counterparts. In principle, an effective regulatory sieve should permit safe products while forming a formidable barrier for those assessed of posing an unacceptable risk to humans, animals and environment.

Molecular farming has become an important area for research and development. Numerous recombinant products for medical and veterinary applications are being produced through genetic manipulation of plants, animals and other living systems including bacteria and fungi. Plant based recombinant products could provide many benefits to society, including drugs and vaccines to respond to diseases such as cancer, diabetes, rabies, foot and mouth disease and the common cold. Since plants used for molecular farming may be producing recombinant products known to be physiologically active in humans or livestock, a regulatory framework is required to safeguard human and livestock health. The regulatory issues associated with plant molecular farming are complex, and involve many sectors of society, including the biotechnology industry, agriculture, medicine, agricultural trade, academia, environmental interest and other civil society groups. The views of these stakeholders as well as the general public should be considered in the development of the regulatory pathway for recombinant products.

The Government of India through the Department of Biotechnology aims to ensure that research and applications in life sciences and biotechnology are guided by a system of monitoring and review that safeguards human and animal health, environment and ensure observance of the highest ethical standards. A scientific, rigorous, transparent, efficient, predictable and consistent regulatory mechanism and protocol for bio-safety and bio-security evaluation and related system need to be followed to meet these objectives. Keeping the above perspective in view, the Department of Biotechnology (DBT) published recombinant DNA safety guide lines in 1990. The guide lines were prepared by the Recombinant DNA Advisory Committee (RDAC). The safety guide lines have been subsequently revised in 1994 and 1998 by DBT based on scientific and technological developments. DBT regulates recombinant research and development in the country by different committees'

mentioned below:

Recombinant DNA Advisory Committee (RDAC): It recommends, from time to time, suitable and appropriate safety regulations for recombinant research, use and applications.

Institute Bio-safety Committee (IBSC): It is mandatory for all research institutions / universities / industries handling microorganism / genetically engineered organisms to constitute IBSC that prepares an up-to-date site emergency plan according to the manuals/guidelines of the Review Committee on Genetic Manipulation (RCGM). This committee also looks into the bio-safety aspects including experimentation and containment issues.

Review Committee on Genetic Manipulation: This committee is based in the Department of Biotechnology. It monitors the safety related aspects in respect of on-going research projects involving genetically engineered organisms/hazardous microorganisms. The Committee also brings out Manuals of guidelines specifying procedure for regulatory process with respect to activities involving high-risk category and controlled field experiments and conducts reviews to ensure that adequate precautions and containment conditions are followed as per the guidelines.

Genetic Engineering Approval Committee (GEAC): This committee functions under the Ministry of Environment, Forest and Wildlife.

The Guidelines developed in 1999 for generating pre -clinical and clinical data for rDNA vaccines, diagnostics and other biologicals, address issues of safety, purity, potency and effectiveness of the project.

The Drug Policy of 2002 deals with the rDNA products where bulk drugs, produced by the use of rDNA technology, requiring *in vivo* use of nucleic acid as the active principles and specific cell/ tissue targeted formulations, require an industrial license for production.

Recombinant DNA Guidelines, formulated by the Department of Biotechnology in 1990, were revised in 1994 for large-scale production and deliberate release of GMOs, plants, animals and products into the environment and shipment and importation of GMOs for laboratory research. It also deals with genetic transformation of green plants, rDNA technology in vaccine development and on large-scale production and deliberate/ accidental release of organisms, plants, animals and products derived by rDNA technology into the environment. Research work has been classified into categories based on the level of the associated risk and requirement for the approval of competent authority. The guidelines give principles of occupational safety and hygiene for large-scale practice and containment, safety criteria and physical containment conditions. They specify appropriate containment facilities depending on the type of organisms handled, potential risks involved and various quality control methods needed to establish the safety, purity and efficacy of rDNA products.

Safety issues

Keeping in view the safety aspects of genetically engineered biologicals, the Government enacted in 1989 rules for the manufacture, use/ import/ export and storage of hazardous microorganisms/ genetically engineered organisms or cells. These rules are applicable to the manufacture, import, export and storage of:

i. Microorganisms and gene-technological products;

ii. Genetically engineered organisms/ micro-organisms and cells and, correspondingly, to any substance and products and food stuff etc. of which such cells, organisms or tissues hereof form part; and

iii. New gene technologies and organisms/ micro-organisms and cells generated by the utilization of such or other gene-technologies and substances and products of which such organism and cell form part.

Under these rules, competent authorities have been identified to ensure implementation of the provisions of the Act and to provide guidelines on ethical and social responsibilities of scientists, institutions, industries, who conduct research, and of those who conduct, fund, administer and regulate work on life sciences.

Environmental issues

'The Indian Environment (Protection) Act of 1986' deals with protection and improvement of environment and the prevention of hazards to human beings, other living creatures, plants and property. Under this Act, the government has the power to take all such measures, as it deems necessary or expedient, for the purpose of protecting and improving the quality of the environment and preventing, controlling and abating damage to the environment, including laying down procedures and safeguards for handling of hazardous substances and carrying out and sponsoring investigations and research relating to problems of environmental pollution. Genetic Engineering Approval Committee approves, from the environmental angle, activities involving large-scale use of hazardous microorganisms and recombinants in research and industrial production and proposals related to release of genetically engineered organisms and products into the environment, including in experimental field trials.

Due to complexity of assessment procedures of environmental impact of release of rDNA organisms/ products, very little information is available. However, it is presumed that most of the biopharmaceuticals will not pose a significant threat to non-target organisms and therefore they will not require additional regulations beyond the regulations for the crop or platform that they will be expressed in and beyond the regulations that will apply to the biopharmaceutical itself. In specific cases where concerns do exist, technologies and management strategies exist to reduce exposure of those biopharmaceuticals of concern to the environment.

Non-target organisms (NTOs) that are at greatest risk due to increased exposure are insects, especially those that are of benefit to and who live within the agricultural setting, and soil microorganisms. The problem with NTOs is that they are sometimes difficult to identify in terms of what could be at risk, and they are very difficult and expensive to study. The risk to NTOs from plant molecular farming depends on a number of elements, including the type and category of recombinant protein that is expressed. While a case-by-case approach for risk assessment is warranted, there are some generalities:

i. Most biopharmaceuticals are going to be proteins with little biological activity (either very specific or none at all), such as vaccines and antibodies.

ii. Most are easily digestible and pose little hazard of toxicity. But not all biopharmaceuticals are going to be the same, and there will be areas where there are concerns about environmental exposure to these pharmaceuticals. Some specific examples include: Biopharmaceuticals that

may be toxic in high doses (such as anticoagulants, hormones and enzymes).

iii. Biopharmaceuticals that persist in the environment (lipophilic).

iv. Biopharmaceuticals that should be contained to keep biologically active products from entering the environment (for example, to limit cross pollination).

Precautions must be taken to limit exposure to the environment through management strategies and technologies, such as inducible genes post harvest (glucocerebrosidase is produced with this technology); product activation post purification, terminator technology to prevent pollen development, and chloroplast transformation to limit gene flow, transgene tracking tools, marker proteins to label specific biopharmaceutical plants and fluorescent protein technology (GFP).

A three-tiered approach can be used.

i. First, look at the product in controlled laboratory settings to see what gets affected by the product itself.

ii. The second tier takes a more ecological setting, such as a greenhouse or enclosed field, and looks for any indication that something is different about this product. Barring any adverse results, a field trial is undertaken.

iii. Isolation/containment strategies include: buffer fields around biopharmaceuticals; secluded or enclosed fields; and greenhouse restrictions. An important consideration is that there must be economic feasibility in terms of the relative risk and benefits.

It is possible that a product gets released that impacts a beneficial organism or attacks an environment in a way that isn't predictable - something that we did not foresee happening. We really don't have any idea of what that situation might be. When you evaluate these products, you need to look at the potential impacts. For certain bioactive products, the prudent thing to do is keep them contained. Products where there is an element of escape, you need to have an acceptable level, and for a benign protein, that could be quite high. Gene flow can be mitigated by confinement measures currently used for research field trials such as guard rows, isolation distances, etc., as well as genetic mechanisms such as male sterility or "Terminator" type technologies. Stakeholders suggested that exposure to NTOs could be mitigated by using tissue-specific or post-harvest inducible promoters.

Ethical issues

Molecular biological research involving humans, animals and plants has provided immense benefits to humankind in the form of vaccines, diagnostics, new drugs and other knowledge for better management of human and animal health. Recombinant DNA technology has opened new vistas for molecular medicine. At the same time, it has also raised questions of social and ethical consequences such as privacy, confidentiality and individual rights to access personal records. Department of Biotechnology developed in 2002 'the Ethical Policies on the Human Genome, Genetic Research and Services'. The objective is to provide guidance to the researchers, ethical committees, institutions, organizations and the public on the conduct of research, based on the recognized ethical principles and values. It addresses issues related to integrity, respect and beneficence; justice; consent; dissemination of research results; gene therapy and human cloning; genetic testing and counseling, genetic privacy and discrimination; intellectual property rights and

benefit sharing; DNA and cell line banking; and international collaboration. Even though these guidelines relate to the ethical policies for genetic engineering research and services, any such research work needs to be approved by the competent authorities, including ethical clearances of the institutions, animal and human concerns, and bio-safety issues. These guidelines provide guidance and also exercise control over the conduct of the life- science scientists.

To prevent the use of biotechnology for biocrimes and bioterrorism, scientists and institutions engaged in all aspects of recombinant research need to abide by a voluntary code of conduct. Scientists should be made aware of the potential risks and concerns relating to science and its wider applications and the ethical responsibilities they shoulder. They should also be aware of and comply with the requirements of international conventions and treaties relevant to their research work.

Concluding remarks

To address the concern of both public and private sector, efforts are under way to establish a single window regulatory mechanism or structure to promote speedy commercialization of recombinant products and processes. A competent National Biotechnology Regulatory Authority (NBRA) is proposed to establish for agriculture products/transgenic crops, pharmaceuticals/drugs and industrial products and transgenic food/feed and transgenic animal/aquaculture. The authority is to be governed by an independent administrative structure to evolve suitable proposals for consideration by inter-ministerial group. A special regulatory cell is likely to be created to build capacity in the country for scientific risk assessment, monitoring and management, to foster international linkages, support biosafety research, to obtain and review feedback from different stakeholders and provide support to industry and R&D institutions to play promotional and catalytic role. NBRA would also evolve new guidelines on transgenic research and product/process development in animal, aquaculture, food, phyto-pharma and environmental application after its set up.

Further Readings

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Meeting of the states parties to the convention on the prohibition of the development, production and stockpiling of bacteriological and toxin weapons and their destruction. Consideration of the content, promulgation, and adoption of codes of conduct for scientists: Indian initiative for code of conduct for scientists prepared by India (2005). Meeting of Experts, Geneva from Dec 5-9, 2005 & June 13-24, 2005, PP, 1-5BWC/MSP/2005/MX/WP.23

CELL CULTURE SCALE UP TECHNIQUES IN BIOLOGICAL PRODUCTION: AN OVERVIEW

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Among the biological products whose production involves animal cell cultivation techniques are viral vaccines (human and veterinary), monoclonal antibodies, cell surface antigens, enzymes, polypeptide growth factors, hormones, immunoregulators, viral bio-insecticides, tumor antigens, cell mass as a product and reconstitution of living skin. The production of these substances is a complicated art requiring knowledge and experience in advanced technologies including cell culture. Cell culture has become one of the major tools used in the life sciences today. Tissue Culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semisolid medium that supplies the nutrients essential for survival and growth. In practice the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. The process of culture of animal cells in an environment outside the tissue (ex vivo) from which it is obtained is called Animal Cell Culture (ACC). Cultured cells are usually described based on their morphology (shape and appearance) or their functional characteristics. There are three basic morphologies:

- Epithelial-like: cells that are attached to a substrate and appear flattened and polygonal in shape.
- Lymphoblast-like: cells that do not attach normally to a substrate but remain in suspension with a spherical shape.
- Fibroblast-like: cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures.

The first vaccine, Jenner's small pox vaccine was produced on skin of living animals and was a very crude preparation. The next vaccine, rabies vaccine was equally contaminated with host proteins. The need for cleaner and safer vaccines led to use of cultured primary cells. In 1907, Harrison cultured frog tadpole spinal chord in a lymph drop hanging from a cover slip of a cavity slide. Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The *Salk polio* vaccine was one of the first products mass-produced using cell culture techniques. This vaccine was made possible by the cell culture research of John Franklin Enders, Thomas Huckle Weller, and Frederick Chapman Robbins, who were awarded a Nobel Prize for their discovery of a method of growing the virus in monkey kidney cell cultures.

Primary Cultures are derived directly from excised, normal animal tissue and cultured either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. They can be maintained *in vitro* only for a limited period of time. Another is continuous culture comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. Morphologically cell cultures take one of two forms, growing either in suspension (as single cells or small free-floating clumps) or as a monolayer that is attached to the tissue culture flasks. (Attached Cell Lines: Ex: Vero,

BHK21 and HeLa etc.).

There are some instances when cell cultures may grow as semi-adherent cells e.g. B95-8 where there appears to be a mixed population of attached and suspension cells. For these cell lines it is essential that both cell types are sub cultured to maintain the heterogeneous nature of the culture.

Cell Culture Scale-up Systems

Vaccine manufacturers were facing the problem of low productivity of cell cultures in particular the anchorage dependent cells. Hence, during 1975-1986 huge efforts were put into developing scalable systems for monolayer cells. In 1967, Van Wezel developed the concept of growing cells on small spheres (100-500micron diameter) that could be put into stirred culture systems. It provided a huge surface area for growth per unit culture volume. In mid 1970s, the first really efficient microcarriers came into market and in 1979; they were used for first time to produce to FMDV vaccine. The availability of porous microcarriers has enhanced perfusion efficiency and protects fragile cells from culture turbulence.

Most tissue culture is performed on a small scale where relatively small numbers of cells are required for experiments. At this scale, cells are usually grown in T flasks ranging from $25cm^2$ to $175cm^2$. Typical cell yield from a T175 flask range from $1x10^7$ for an attached line to $1x10^8$ for a suspension line. However, exact yields will vary depending on the cell line. It is not practicable to produce much larger quantities of cells using standard T flasks, due to the amount of time required for repeated passaging of the cells, demand on incubator space and cost.

When considering scaling up a cell culture process there is a whole range of parameters to consider which will need to be developed and optimized if scale-up is to be successful. These include problems associated with nutrient depletion, gaseous exchange particularly oxygen depletion and the build up of toxic by-products such as ammonia and lactic acid.

Scale-up Solutions

There are two different categories of scale-up systems:

- Systems suitable for attached or anchorage dependent cells
- Systems suitable for cells that grow in suspension

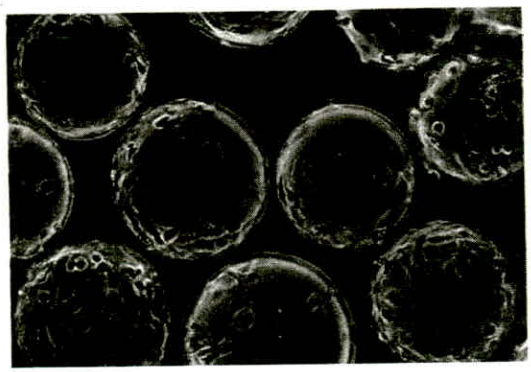
Systems for anchorage dependent cells

***Roller Bottle Culture*:** This is the method most commonly used for initial scale-up of attached cells also known as anchorage dependent cell lines. Their use for large-scale culture was first described by Gey in 1933. Roller bottles are cylindrical vessels that revolve slowly (between 5 and 60 revolutions per hour) which bathes the cells that are attached to the inner surface with medium. The size of some of the roller bottles presents problems since they are difficult to handle in the confined space of a microbiological safety cabinet. Recently roller bottles with expanded inner surfaces have become available which has made handling large surface area bottles more manageable, but repeated manipulations and subculture with roller bottles should be avoided if possible. A further problem with roller bottles is with the attachment of cells since as some cells lines do not attach evenly. This is a particular problem with epithelial cells. This may be partially

overcome a little by optimizing the speed of rotation, generally by decreasing the speed, during the period of attachment for cells with low attachment efficiency. A number of modern biological medicines have been produced in large-scale roller bottle facilities including a number of vaccines.

***Stacked plate system*:** These culture systems contain a number of flat culture surfaces stacked in parallel one above the other within a single unit. They do not require agitation. Media and other solutions are added through an access port and distributed between the units by tilting the unit. They have been used for production of measles, mumps, rubella and polio vaccine. But the demerits are no direct access to culture surface and they are made up of rigid plastic and thus are susceptible to damage.

***Microcarriers*:** Using these tiny beads (20µm dia.) surface area in excess of 30 cm^3/cm^3 of culture medium are easily attainable for use in simple batch culture and higher values for fed batch or perfuse culture. These small beads are 30-1005m in diameter and can be made of dextran, cellulose, gelatin, glass or silica, and increase the surface area available for cell attachment considerably. Originally DEAE Sephadex A-50 beads were used but the density of positive charge on these beads was too high for optimum cell attachment and growth. The material to be used must not be toxic to the cells and must not be inhibitory to cell growth. A recent advance has been the development of porous micro-carriers which has increased the surface area available for cell attachment by a further 10-100 fold. The surface area on 2g of beads is equivalent to 15 small roller bottles.



Vero cells growing on microcarriers

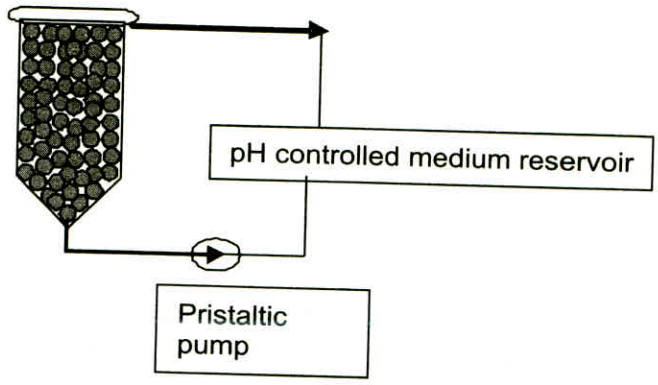
Merits: Culture of cells can be carried out in the same type of equipment that is employed for homogenous stirred culture of cells growing in suspension. This system permits direct sampling and observation of cells.

***Packed bed system*:** Glass beads are used in this system. It is best suited to the harvesting of a secreted product over a period of time.

Schematic diagram of packed bed system

Systems for suspension culture

***Spinner Flask Culture*:** This is the method of choice for suspension lines including hybridomas and attached lines that have been adapted to growth in suspension e.g. HeLa S3. Spinner flasks are either plastic or glass bottles with a central magnetic stirrer shaft and side arms

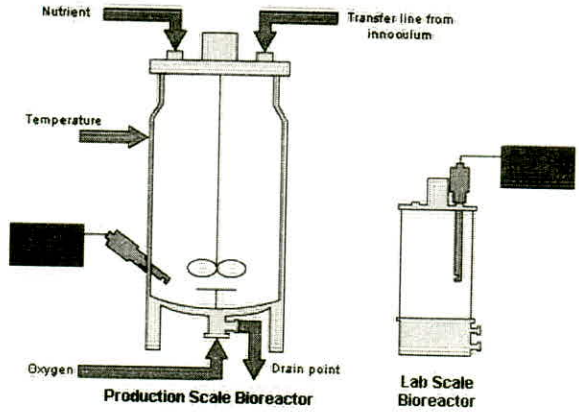


for the addition and removal of cells and medium, and gassing with CO_2 enriched air. Inoculated spinner flasks are placed on a stirrer and incubated under the culture conditions appropriate for the cell line. Cultures should be stirred at 100-250 revolutions per minute.

***Shaker flasks*:** Erlenmeyer style flasks are secured to shaker apparatus that mixes the contents of the flask and keep the cells in suspension. They are used for small to moderate volumes of cell having high oxygen requirement.

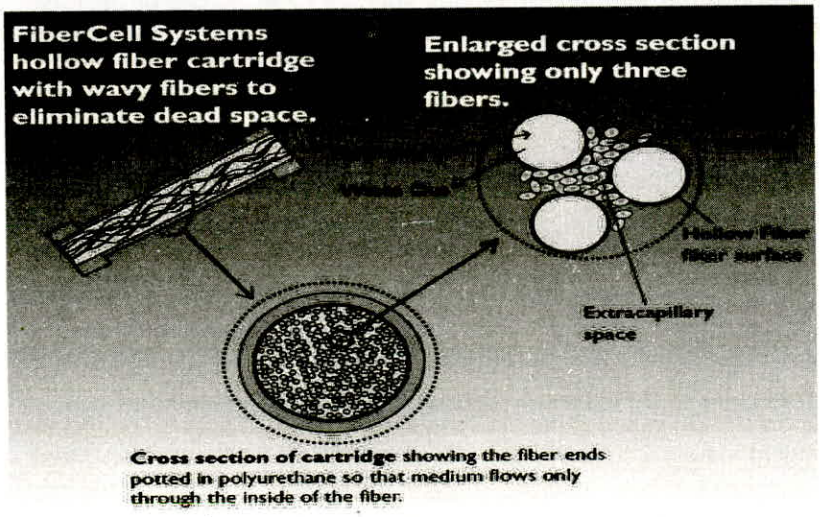
***Culture bags*:** They are consisting of sterile single use non-glass permeable pillow shaped bag mounted on a rocking apparatus. The bag is inflated with CO2/air mixture suitable for the medium to be used, and then the medium is introduced into the bag after which the cells are added. The bag retains a substantial headspace of CO2/air and it comes fitted with a fill tube, harvest tube. Inlet filter, exhaust filter, sampling port, constant pressure relief valve and ports for in-situ pH and dissolved oxygen probe. Advantages are simple principle, low shear environment and disposable; whereas the disadvantages include problem with in-situ sensors and resultant inability to monitor the culture environment in real time. However, this problem can be overcome with a newly developed system having in-situ pH and temperature sensor interfaced with a controller that allows feed back control.

***Fermentors*:** For industrial scale production of animal cells by far the commonest method is submerged culture in stirred tank or airlift Fermentors. Both fed batch as well as perfusion process can be employed in Fermentors.



Other Scale up Options

- *Dialysis tubing culture* system used for generating monoclonal antibodies for research purpose only.
- *Hollow-fibre system* for anti-body and recombinant protein production: The hollow fiber filters provide a large amount of surface area for cell attachment while concentrating secreted proteins or antibodies in the small volume of the cartridge. Hollow fibers are small tube-like filters approximately 200 microns in diameter whose molecular weight cut-off can be between 5kDa and 0.1μm. These fibers are sealed into a cartridge shell so that cell culture medium pumped through the end of the cartridge will flow through the inside of the fiber while the cells are grown on the outside of the fiber. Since the cells are attached to a porous support (the hollow fiber) rather than non-porous plastic dish nutrients are delivered from the bottom layer of cells on upwards. Splitting of the cells is not required and cultures can be maintained for many months of continuous production. If the secreted protein can be retained in the extra-capillary space it will accumulate to a concentration of up to 100 times higher than with conventional flask or roller bottle culture. Hollow fibers also provide a tremendous amount of surface area in a small volume. Cells grow on and around the fibers at densities of greater than 1 X 108 per ml.



Cross section of cartridge showing the fiber ends potted in polyurethane so that medium flows only through the inside of the fiber.

Hollow fiber cell culture is the only means to culture cells at in vivo like cell densities. Cell culture at high densities has the following benefits:

- Reduces serum requirements and facilitates adaptation to serum-free medium
- Increases the concentration of secreted product by 10 to 100 times
- Viral and parasitic infections proceed rapidly
- Low shear environment

***Disadvantages*:**

- Very less suited to the culture of attachment dependent cells
- Not possible to remove a sample of cells during course of a culture
- Suitable only for production of secreted products but not suited to production of biomass

CELL Line flask: It is a two compartment bioreactor in which cells are separated from bulk of medium by a semi-permeable membrane. In this cells can be easily removed from the vessel but there is only a limited degree of scale-up.

***Mini PERM vessels*:** It is two compartment roller bottles in which the main reusable compartment contains a bulk of medium and is separated from the disposable cell containing compartment. They are useful in producing small quantities of monoclonal antibodies.

***Encapsulation*:** It is available in different formats including hollow spheres of polylysine or cellulose or alginate spheres and agarose beads.

Ceramic matrix: A ceramic matrix having a high degree of surface area per unit volume is shown to have significant utility in the large-scale culture of animal cells. The surfaces of the ceramic provide for the adhesion and growth of a wide variety of cells to densities equal to or greater than obtained with other methods such as roller bottles or microcarriers. Utilizing an automated system controlling pH and dissolved oxygen, scale-up from 0.9 m^2 to 18.5 m^2 of surface area can be accomplished with no losses in efficiency of surface utilization. The density of Vero cells after 7-8 days culture under standard conditions averaged 6.6 cells/cm^2 for each size of ceramic.

***Bioreactor*:** The next stage of scale up for both suspension and attached cell lines is the bioreactor that is used for large culture volumes (in the range 100-10,000 liters). For suspension cell lines the cells are kept in suspension by either a propeller in the base of the chamber vessel or by air bubbling through the culture vessel. However, both of these methods of agitation give rise to mechanical stresses. A further problem with suspension lines is that the density obtained is relatively low; in the order of $2x10^6$ cells/ml.

Nano-Liter Bio-Reactor: *long-term mammalian cell culture at nanofabricated scale for growing and maintaining populations of up to several hundred cultured mammalian cells in volumes three orders of magnitude smaller than those contained in standard multi-well screening plates. NBR incorporates a culture chamber, inlet and outlet ports, and connecting micro-fluidic conduits. The input and outlet ports enables the supply and withdrawal of culture medium into/from the culture chamber (10-100 nL volume), as well as cell seeding. The employment of NBRs for mammalian cell culture opens a new paradigm of cell biology.*

Micro-fluidic PDMS (polydimethylsiloxane) bioreactor *could provide suitable environments for cell culture because of the larger surface-to-volume ratio and fluidic behavior similar to the environments in vivo. Such micro-fluidic environments are now used to investigate cell-to-cell interactions and behaviors in vitro, emulating situations observed in vivo, for example, micro-scale blood vessels modeled by micro-fluidic channels.*

The choice of system for scaling up animal cell culture for biological production is complex

with a number of systems available. Each system has its own merits and demerits for different cell types and products. Taking into account not only the biological and engineering characteristics of system but the cost and regulatory implications various options can be investigated for further use.

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RECENT TRENDS IN THE DEVELOPMENT OF VETERINARY VACCINES

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The programmes of immunization have led to the elimination and/or control of several different infectious diseases, including smallpox, polio, measles, mumps, rubella, Haemophilus influenzae type B disease, pertussis, tetanus, and diphtheria in case of human beings. Similarly, in case of animals, the diseases like foot and mouth disease, rabies and rinderpest have been eradicated form different parts of the world, owing to mass eradication campaign launched in selected areas. Development of these vaccines was made possible due to the technologies of the 19th and 20th centuries. These include, inactivation by heat, chemicals, and irradiation to produce a killed vaccine, vaccination with a serologically related virus, and attenuation by tissue culture passage to produce live vaccines with substantially reduced virulence. However, the vaccines of 21st century are being developed by improvements in these basic techniques and through the use of new technologies based on the expanding understanding of the immune response. New, and still unmet, targets for human vaccine development include some of the more difficult infectious agents, such as cytomegalovirus, human immunodeficiency virus (HIV) and severe acute respiratory syndrome coronavirus; bacteria, such as Pseudomonas aeruginosa, Neisseria gonorrhea, or Mycobacterium tuberculosis; and parasitic diseases, such as malaria or hookworm disease.

In case of veterinary medicine, although vaccines are available for most of the infectious diseases, the emergence of new strains of infectious agents like avian influenza and FMD is a major challenge in the development of animal husbandry and therefore, needs constant search for alternative strategies for developing more effective vaccines. Besides this, the focus of research in the present time is on development of multi-component vaccines; multivalent vaccines and marker vaccines that can easily differentiate the vaccinated population from infected ones. The development of such vaccines, which can provide life long immunity in single shot vaccination is another target area for vaccinology research.

The vaccine presently used for prophylaxis of viral diseases like FMD is an inactivated whole-virus preparation that is formulated with adjuvant prior to use. The countries where the FMD is highly endemic are also maintaining concentrated antigen stored in the gaseous phase of liquid nitrogen (Doel, 2003) wherein, the antigen is stable for a longer period of time than the formulated vaccine (Doel and Pullen, 1990). The concentrated vaccine antigen contain antigen against a number of virus serotypes and serve as an immediate source of vaccine in case of any epidemic. Although the introduction of the killed FMD vaccine has been extremely successful in reducing the number of disease outbreaks in many parts of the world where the disease is enzootic, there are a number of limitations with its use in emergency control programs. Especially, high-containment facilities are required for the production of these vaccines, because these vaccines are mostly concentrated cell culture supernatants from FMDV-infected cells.

The vaccinated animals sometimes develop antibody responses against the contaminating proteins, in addition to the viral structural proteins, making it difficult to reliably distinguish vaccinated from infected or convalescent animals with currently approved diagnostic tests. The vaccine does

not induce rapid protection against challenge by direct inoculation or direct contact. Thus, there is a window of susceptibility of vaccinated animals prior to the induction of the adaptive immune response. The vaccinated animals can also become long-term carriers following contact with FMDV owing to the limitations associated with these types of vaccines. An alternative in the form of marker vaccine development has been adopted, where infectious virus is not required to elicit the immune responses and diagnostic tests that are based on NS proteins are used to differentiate the vaccinated form non-vaccinated animals.

Based on the availability of increasing information about the genomics and proteomics data of different infectious organisms, a number of alternative vaccine strategies have been employed. These include the use of individual proteins either isolated from purified virus or produced by recombinant DNA techniques (Bachrach *et al.*, 1975; Kleid *et al.*, 1981); or the use of immunogenic viral protein derived peptides (Strohmaier *et al.*, 1982) or chemically synthesized immunogenic peptides peptides (Bittle *et al.*, 1982; DiMarchi *et al.*, 1986). Other strategies for the development of vaccines include the use of live vectors expressing fusion proteins (Kit *et al.*, 1991); or inoculation with DNA expressing immunodominant epitopes, either alone (Wong *et al.*, 2000) or co-administered with DNA encoding cytokines like IL-2 (Wong *et al.*, 2002).

Advances in the field of transgenic technology have also made available immunogenic proteins in the different plants like Lucerne and pigeon pea. The H protein of PPR virus and VP1 proteins of FMDV has been expressed in the plants under the control of TMV vector (Wigdorovitz *et al.*, 1999) and the transgenic edible vaccine has shown protection in laboratory animals. All of these strategies present a limited subset of viral immunogens to the vaccinated animal and although they often induce high titers of neutralizing antibodies, they do not always achieve protection against virus challenge in livestock (Mulcahy *et al.*, 1990). The immunogenicity of these subunit vaccines is mainly due to their ability to present the sequential epitopes to the immune system of the host. Only a few representative vaccine technologies, which have gained momentum in the present time, have been discussed in details in this paper.

DNA Vaccines or Genetic Vaccines:

Deoxyribonucleic acid (DNA) vaccines or genetic immunization, are those, which consist of genes encoding the antigen rather than the antigen itself. The development of DNA vaccines grew from efforts to generate MHC class I restricted CTL responses by capitalizing on the understanding of different intracellular antigen processing pathways. DNA vaccination is a novel vaccine technology that has great potential for reducing infectious disease and cancer- induced morbidity and mortality in animals and human subject, worldwide. Ever since their inception, DNA vaccines have been used to stimulate protective immunity against many infectious pathogens, malignancies, and autoimmune disorders in animal models. Wolf *et al.* (1990), initially demonstrated that unformulated plasmid DNA derived from bacteria, encoding a marker protein and under the control of a promoter capable of functioning in mammalian cells, could be taken up by muscle cells in the mice following direct intra muscular injection and resulted in synthesis of the encoded protein. However, the low amount of protein produced, the apparent lack of transfection of professional antigen presenting cells and the absence of any replicative step, made it surprising that intra muscular immunization of mice with plasmid DNA encoding a viral protein could generate CD8+, CTL, as well as, antibody responses (Ulmer *et al.*, 1993).

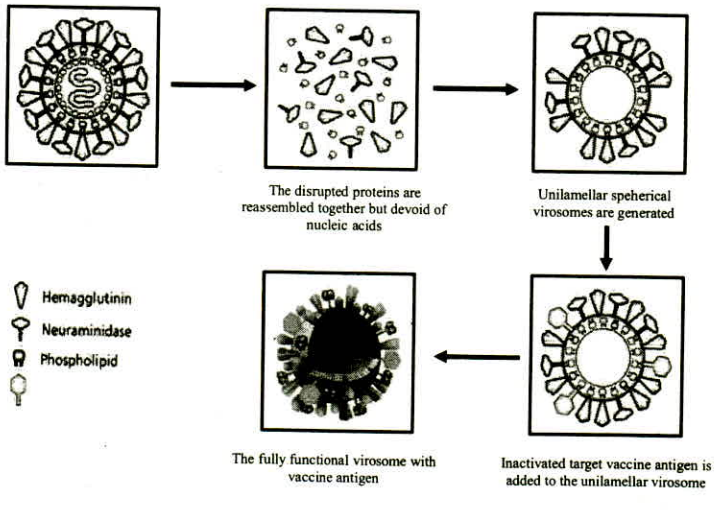
The plasmid DNA encoding a polypeptide protein antigen is introduced into a host, where it enters

host cells and serves as an epigenetic template for the high-efficiency translation of its antigen. This is followed by an immune response, which is mediated by either the cellular and/or humoral arms of the immune system. This immune response is specific for the plasmid-encoded antigen wherein, the "professional" APCs play a dominant role in the induction of immunity by presenting vaccine peptides on MHC class I molecules, following direct transfection or "cross"-presentation, and MHC class II molecules after antigen capture and processing within the endocytic pathway. The vaccine response to the DNA vaccines can be manipulated according to many immunization parameters, including the method of vaccine delivery, presence of genetic adjuvants, and vaccine regimen. Although, the genetic vaccine strategies have been tried for the past two decades, further analysis is required to determine their ultimate efficacy and safety target hosts. The DNA vaccine technology has acquired a strong foothold in the field of experimental immunotherapy, and it is hoped that it will eventually represent the next generation of prophylactic and therapeutic vaccines.

Empty viral capsids / Virosome Vaccines:

In another approach, vaccines have targeted immunogens that contain the entire repertoire of immunogenic sites present on intact virus but lack infectious nucleic acid (Moghimi *et al.*, 2005). This strategy involves the molecular cloning of the regions of the viral genome necessary for the synthesis, processing, and assembly of the viral structural proteins into empty viral capsids. In case of FMDV-infected cell culture systems these structures are naturally produced, are antigenically similar to virus particles, and are as immunogenic as virions in animals. The animals inoculated with this type of vaccine could be easily distinguished from infected or convalescent animals by using currently approved technology, since the regions of the genome coding for the NS proteins used in the diagnostic assays to detect infection are not present in the empty capsid cDNA construct. In initial studies, FMDV capsid structures were expressed in Escherichia coli or in recombinant baculovirus-infected cells and inoculated into animals. Although these products did offer some protection, they did not reach the efficacy of the current inactivated whole-virus vaccine because only small amounts of antigen are obtained by this method (Grubman and Baxt, 2004).

Schematic representation of the development of the empty viral capsid / virosome vaccines



Expression Library Immunization:

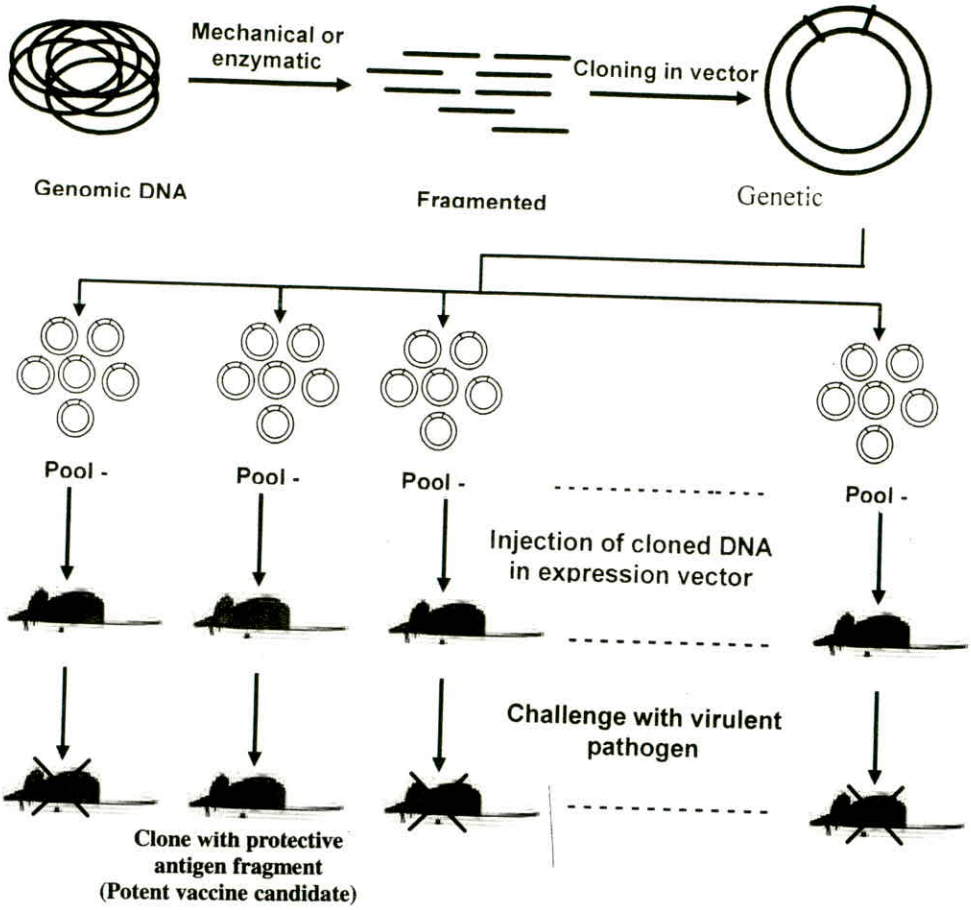
The Expression library immunization (ELI) protocol developed by Barry *et al.* (1995) helps in screening of any given genome to identify potential vaccine candidates. The basic concept of ELI can be applied to screen sequenced or un-sequenced genomes of infectious agents. In the ELI, the pathogen's genome is reduced in a relatively unbiased way to only a few antigens that are responsible for eliciting protective immune responses in the target host. The essential concept in adopting this approach is to employ the host immune system to select the best vaccine candidates against a particular disease. In this approach the entire genome of a pathogen (either bacterial, viral, parasitic, or fungal) is cloned into genetic immunization vectors under the control of a eukaryotic promoter to create a library that would express all the possible open reading frames (ORFs) of a pathogen. The purified plasmid DNA from this library is inoculated into animals, usually in sub-genomic pools of clones, to induce immune responses against the cloned antigens. The immunized animals are then challenged with the pathogenic organism to identify the clones that induced protective immunity. These antigens are then further delineated and de-convoluted to map the exact length of protective antigens. The ELI technique provides a rapid screening protocol for an entire genome and the readout of screening is identification of protective antigens, which is the end goal for any vaccine development project.

The genetic immunization is increasingly becoming a leading technology for screening potential vaccine candidates against both human and animal diseases, with the sole aim of developing subunit vaccines. The genetic vaccines have several advantages over traditional methods. Take for example, the subunit vaccine, wherein, only the antigen or antigens that produce an effective immune response are included, to reduce any unintended responses. However, since the inoculating agent is DNA, the purification and handling of the genetic vaccines are simpler than those for traditional vaccines. The genetic vaccines also do not require "cold chain" facilities to keep the gene vaccine viable, which is not true for live attenuated vaccines. Besides this, the genetic vaccines gain direct entry in to cells via a delivery process that mimics natural infection, where pathogens can enter the host cells and does not require unwanted replication that is associated with live attenuated vaccines. Above all, the delivery of a very small amount of a genetic vaccine (nearly 100 ng) can elicit a strong protective immune response in the target host (Barry and Johnston, 1997).

A diagrammatic representation of the Expression Library Immunization (ELI) protocol for identifying the immunodominant domains in the genome of infectious agent for development of subunit vaccine

Synthetic peptide vaccines:

The antigenic reactivity of any protein is located in those regions of the molecule that are recognized by the binding sites or paratopes of certain immunoglobulins. The region of an antigen that is recognized by a paratope is called an antigenic determinant or epitope. The epitope nature of a cluster of amino acids in a protein is recognizable only through the binding of an immunoglobulin. The epitopes which can be classified as either continuous or discontinuous are not intrinsic feature of a protein existing independently of its paratope partner, but a relational entity that can be defined only in an operational sense by the binding of a complementary paratope (Van Regenmortel, 1989). The continuous epitope is any short, linear peptide fragment of the antigen that is able to bind to antibodies raised against the intact protein. Because the peptide fragment usually does



not retain the conformation present in the folded protein and mostly represents only a portion of a more complex epitope, it shows less reaction with the antiprotein antibodies. Not every residue in a so-called continuous epitope is necessarily a contact residue interacting with the paratope. The approach used to find out the contribution of individual residues in a peptide to the binding interaction is by replacing each residue in turn by the other 19 possible amino acids (Getzoff *et al.*, 1988).

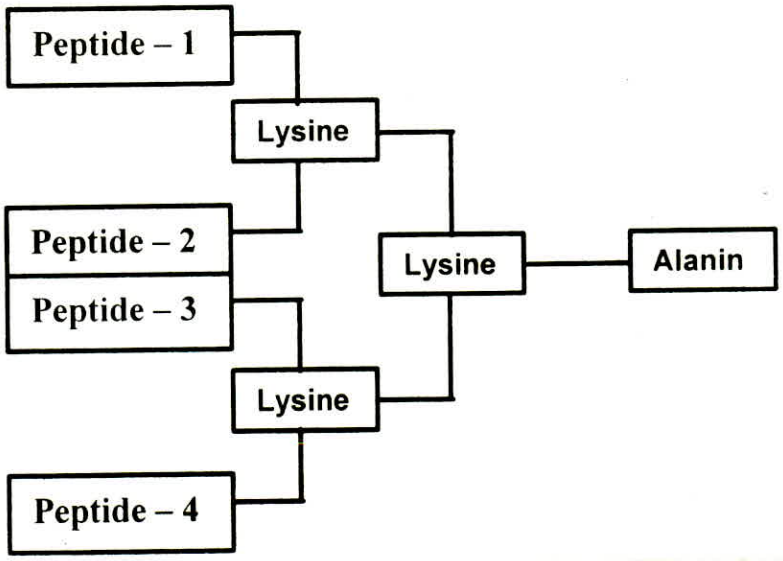
With the information available on the essential amino acid in any reactive epitope of a protein, the development of synthetic vaccines become possible, which can overcome several problems incurred with the use of conventional vaccines consisting of either killed or live attenuated disease-causing organisms. Notwithstanding the tremendous achievements obtained with such vaccines in reducing both incidence and morbidity of many infectious diseases, considerable obstacles that interfere with their effective application are often encountered, such as high restriction to strain and type specificity of the infectious agent due to continuous antigenic variations in viruses and parasites; MHC restriction of the immune response; difficulties in tissue culture growing of the organism as well as production and storage of many vaccine preparations; biohazard in both production and use of vaccines against lethal infections, etc. Because of these complications there are still many viral and parasitic diseases for which no effective vaccines exist, and ap-

proaches are sought to circumvent the problems of conventional vaccines.

The use of synthetic peptides for vaccination is one alternative that is vigorously being explored. Its attractiveness is the simplicity of the approach, the information it brings to the molecular understanding of the immune response required for protection, and the considerable practical advantage that such products could offer. These vaccines contain a relatively small peptide or peptides, which have been shown to constitute epitopes of the organism that elicit a protective immune response. In principle, by selection of only those epitopes that confer an effective immunity, it should be possible to exclude the epitopes responsible for deleterious immune responses. Furthermore, as they are chemically defined and do not contain infectious material, they should be devoid of any risk as biohazard factors.

In recent times, the multiple antigenic peptides (MAPs) corresponding to the immunodominant epitopes of the viral and bacterial proteins are synthesized and are used successfully for the development of peptide vaccines. These MAPs have shown to be promising antigens, both for detection of infectious organism specific antibodies in clinical sera, and also for generating epitope specific response in the host (Shrivastava, 2006). The MAP format of the short linear peptides helps in the multimerization of the epitopes, thereby generating a molecular mass, which is to the tune of about 15kDa. This higher mass of the single epitope in the multimeric branched; MAP format essentially triggers the immune system to respond in the form of antibodies.

Schematic representation of the Multiple Antigenic Peptide



The synthetic peptide vaccines are proving to be more safe and thermo stable reagents, as they are free of any live virus handling. In a search for the alternative vaccine techniques for the use of VP1-derived natural peptides (Strohmaier *et al.*, 1982) or chemically synthesized VP1 peptides have been used successfully (Pfaff *et al.*, 1982; Nargi *et al.*, 1999). The first synthetic peptide vaccine providing protective immunity was developed for the canine parvovirus infection in dogs (Langeveld *et al.*, 1994), wherein, the antigenic sites representing the amino-terminal region of viral protein VP2 were mapped and synthetic peptide corresponding to this epitope was found to

elicit protection on virulent virus challenge. It was also suggested that just like the marker vaccines, it was possible to discriminate between vaccinated dogs that have not been exposed to the virus and dogs that have been infected with the virus, provided the synthetic peptide vaccine was designed judiciously.

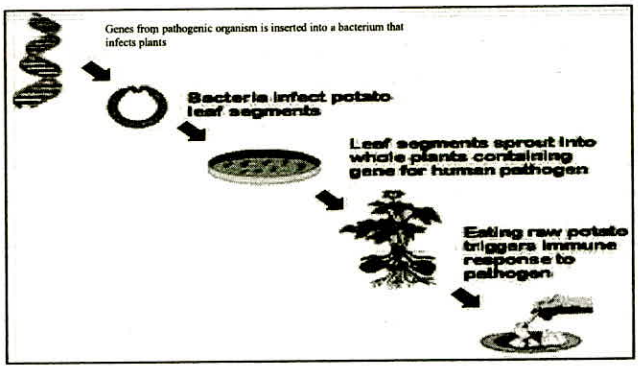
Several advantages can be envisaged for vaccines based on synthetic peptides if their production materializes. It will be possible to synthesize more complex antigens incorporate peptides consisting of the relevant epitopes several different viruses and/or bacteria in their structure, thus leading to multivalent vaccines that are simultaneously effective toward all these pathogens. With the design and synthesis of lapidated MAPs, it might be possible to include built-in adjuvanticity in the vaccines and thus overcome a serious problem in the production of existing vaccines. It may also be possible to take advantage of our knowledge about the linkage of immune response and the major histocompatibiity complex for the design of synthetic vaccines tailored to individuals according to their HLA type. The prospect of constructing successful synthetic peptide vaccines grows as our knowledge in all areas of immunology increases, and with the pace of research in the field of vaccinology, the dream for developing an ideal vaccine might become a reality in the not-too distant future.

Plant Derived Genetically Modified Vaccines (Edible Vaccines)

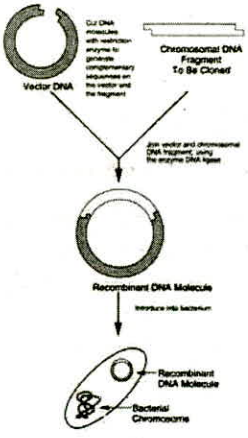
The advances in field of Modern biotechnology, has led to re-birth of interest in obtaining new medicinal agents from botanical sources. Through genetic engineering, plants can now be used to produce a variety of proteins, including mammalian antibodies, blood substitutes, vaccines and other therapeutic entities (Fischer and Emans, 2000). Recently, the production of foreign proteins in genetically engineered plants has become a viable alternative to conventional production systems such as microbial fermentation or mammalian cell culture. The genetically engineered plants, acting as bioreactors, can efficiently produce recombinant proteins in larger quantities than those produced using mammalian cell systems (Daniel *et al.*, 2001). The plant-derived proteins are particularly attractive, since they are free of human diseases and mammalian viral vectors. Large quantities of biomass can be easily grown in the field, and may permit storage of material prior to processing. Thus, plants offer the potential for efficient, large-scale production of recombinant proteins with increased freedom from contaminating human pathogens.

The transgenic pharmaceutical plants are primarily modified by the introduction of novel gene sequences under the control of suitable promoter, which will either help in tissue specific expression of proteins or results in generalized expression of foreign proteins in the modified plants. The 'designer' proteins or peptides produced in the plants possess therapeutic value themselves. These proteins may be used as precursors in the synthesis of medicinal compounds, or may serve as technical enzymes in pharmaceutical production. In case of veterinary medicine the expression of immunogenic proteins of FMD virus, rinderpest virus and PPR virus have been attempted in the fodder plants. Although there was high level of expression of foreign proteins in the genetically engineered plants, the desired protection on pathogen challenge could not be achieved.

Schematic representation of production of edible vaccines by expressing foreign proteins in plant system



Recombinant Vaccines:



The advances in field of recombinant DNA technology, has made it possible to express the foreign proteins in different expression systems. With the availability of high capacity vectors, it has also become possible to accommodate the large proteins in the expression systems. Besides this, with the advance research in the field of analytical biochemistry, various purification techniques are available to harvest the purified proteins, which can be directly used as sub-unit vaccines or development of diagnostics. Given as under is a comparative account of the merits and demerits of different expression systems, which are commonly used to generate high quality purified proteins. Schematic representation for the production of recombinant proteins in the bacterial expression system

Bacterial Expression Systems

Sl. No.	Advantages	Disadvantages
1	The rate of growth is very high and the recombinant proteins can be produced from the *E. coli* culture transfected with the plasmid containing desired gene in a period of about 8-12 hours	In the prokaryotic systems there is no provision of post-translational modifications of proteins like glycosylation and removal of signal peptides.
2	The yield of recombinant proteins is about 50-500mg/L of bacterial culture.	The proteins with a molecular mass greater than 50kDa cannot be expressed
3	The media used in the cultivation of bacteria is generally very cheap and therefore, the cost incurred in the production of recombinant proteins by expression in bacterial systems is very low.	Some times the expression of foreign eukaryotic proteins in the prokaryotic system leads to toxicitiy in cells resulting in decrease of overall yield of these proteins. This is often true for certain enzymes and toxins.
4	The large scale production of recombinant proteins in the prokaryotic system, often results in low fermentor costs due to rapid growth of cells and less growth requirements	In the prokaryotic systems the expression of proteins containing rich disulphide bonds is not possible.

Yeast Expression Systems

Sl. No.	Advantages	Disadvantages
1	The yeast grows very rapidly and can produce recombinant proteins	The yeast expression systems has chaperonins to help proper folding of
2	The yields of recombinant proteins ranges from 50-5000 mg/L	The yeast expression systems are equipped with adequate post-translational machinery to effect the glycosylation of proteins and removal of signal peptides
3	Just like in the bacterial expression systems, the cost of growth media is lower in the yeast expression systems also.	In the yeast expression system, the production or proteins with higher molecular mass i.e., greater than 50 kDa is possible, if suitable vectors are used.
4	Scaling up of the product is possible because the cost of fermenter generated recombinant proteins is very less	The yeast expression can produce adequate micro-environment for the formation of disulphide bridges in the proteins

Bacculovirus Expression Systems

Sl. No.	Advantages	Disadvantages
1	Can express large recombinant proteins, of the size greater than 50 kDa. At the same time, the yield of the recombinant proteins is also very high.	The bacculovirus expression system grows very slowly, i.e., and requires about 10-12 days for yielding the proteins.
2	In this system, there proper glycosylation & signal peptide removal in the recombinant proteins	In case of insect origin cell lines, the growth is sustainable only for 4-5 days
3	This system has a battery of chaperonins to help proper folding of "tough" proteins.	The set up of any bacculovirus expression system is time consuming, not as simple as yeast or bacterial system

Mammalian Expression Systems

The production of improved prophylactics for prevention of diseases has been a continuous process and with developments in the field of science and technology, ever increasing number of efforts are being made to develop more safe, potent and cost effective vaccines for different animal diseases. The future of vaccine production process lies in the success of developing vaccines which provide long-term immunity and are easy to administer. In case of animal science, the new generation vaccines like DNA vaccines, edible vaccines and synthetic peptide vaccines will prove to be very successful, when the present hurdles of bulk production are overcome.

Sl. No.	Advantages	Disadvantages
1	The expression of large proteins (>50 kDa) is possible	The establishment of mammalian expression system and selection of recombinant clones is time consuming
2	Equiped with correct glycosylation & signal peptide removal, therefore, it generates authentic proteins	Cell culture is only sustainable for limited period of time (every mammalian cell has unique growth and turn over time)
3	Chaperonins are available for proper folding of "tough" proteins	The entire set-up is very time consuming and costly as well as, provides less yields.

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SESSION VIII

Production Biotechnology and their commercial aspects

Chairman : **Dr. Balakrishnan**

Co-Chairman : **Dr. A.K. Misra**

Rapporteur : **Dr. Shiv Prasad**

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COMMERCIAL USE OF MODERN REPRODUCTIVE TECHNOLOGIES IN THE IMPROVEMENT OF ANIMAL PRODUCTION

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Introduction

The vast livestock genetic resources have been an integral component of Indian Agriculture. During recent years, due to commercialization of agriculture, more emphasis has been given to cash crops, which contribute little to quality animal food. As a result, livestock breed sustainable on agriculture byproducts have to become relatively uneconomical and are being replaced by more productive indigenous and exotic breeds from different environment (Madan, 2007). Then present trends indicate that animal protein and animal products requirement would rise faster than cereals in the consumption pattern both in India and globally mainly due to increase in income and need for quality food. This could be achieved using gene technologies in sustainable production systems while maintaining environment and addressing concerns of animal bio-diversity, quality of products, human and animal health and welfare. Reproductive biotechnologies, such as Artificial insemination (AI), embryo transfer (ET), *in vitro* Fertilization (IVF), cryopreservation of embryos, sexing, cloning, transgenics, and stem cell technology, singly or in combination, can increase reproduction rates and play prominent role in the genetic improvement of livestock and their conservation (Misra and Prasad, 2005). Following the birth of the first ET cattle and buffalo calves in India in 1988, several research institutes and Universities are now actively engaged in research and production in various aspects related to embryo biotechnology and the stage is set to exploit proven technologies to accelerate genetic improvement of livestock. Accordingly, this paper deals with the available biotechnologies, which can be used commercially to enhance the potential of animal production.

Reproductive Biotechnologies and its commercial use

The major objective of application of biotechnology in animal reproduction is to increase reproductive efficiency and increased rate of animal genetic improvement, thereby increasing the output from the animal production system. The available biotechnologies which are commercially viable for enhancing productivity of animals are as follows:

2.1 Artificial Insemination (AI)

AI is the first generation of reproductive technologies, which is still considered to be the most efficient and cost effective tool to disseminate superior quality genetic material among breeding population to augment the productivity of milch cattle at a faster rate. The long term preservation and storage of semen has further broadened the dimensions of AI by enabling its world wide distribution, at a very low cost.

Use of AI coupled with progeny testing, resulted in manifold increase in milk production per cow per year in several western countries. In addition, each bull is able to produce a Large number of daughters in a given time thus enhancing the efficiency of progeny testing of bulls. Compared to natural mating, high intensity and accuracy of selection following AI can lead to a four-fold increase in the rate of genetic improvement in dairy cattle (Van Vleck, 1981). This technique has also

enabled, introduction of new genetic material by import of frozen semen at negligible cost compared to the import of live animals, use of frozen semen even after the death of bull and control of venereal diseases.

In India, AI was introduced in early forties, however, its wide application started in late fifties only. Initially the AI was used to spread improved indigenous breeds, which was followed by the introduction of crossbreeding in late sixties (Bhat and Taneja, 1998). In terms of breeding infrastructure, we are the largest in the world with 64 frozen semen stations and more than 60,000 AI centres. Of these, 54 functional frozen semen stations are producing 37 million frozen semen straws. Semen production in the country has been increased from 22 (1999-2000) to 37 million straws (2004-2005), and AI has increased from 20 to 34 million. As per the impact analysis report submitted by NABARD, overall conception rate has increased from 20 to 35% in four states (Andhra Pradesh, Haryana, Uttranchal, and Madhya Pradesh) and coverage of the breedable bovine population has increased from 16 to 28%. The present coverage of AI in cattle and buffalo is only about 20 and 10% of all breedable animals, respectively, with the conception rate of about 20% in cattle and 10% in buffalo. However, there are several institutions where the conception rate following AI is 60% or more, which largely depends on quality of semen, accurate heat detection, skills of inseminator and timely insemination.

2.2 Cryopreservation of gametes

Cryopreservation allows virtually indefinite storage of biological material without much deterioration over a time scale for at least several thousand years (Mazur,1985). Semen, ovum and embryos of cattle are frozen routinely and stored in respective banks. Dedicated freezing media, equipment for semen collection, processing, freezing and AI have been developed and are available commercially for large number of mammalian species (Ruane and Sonnino, 2006).

2.2.1 Cryopreservation of sperm

The landmark success in cryopreservation of semen using glycerol as a cryoprotectant (Polge *et al.*, 1949), made profound impact on the use of frozen semen for AI. The basic technique in this experiment has been improvised over the years and frozen semen of cattle, buffalo and other species of livestock is produced on commercial scale in several countries.

In the XIth Plan, target for frozen semen production is pegged at 70 million doses annually as against present production of about 37 million doses. With the aggressive campaign for pedigree breeding and AI, it is estimated to increase the acceptability of artificial insemination by the farmers to about 40% in indigenous cattle and 100% in crossbred cattle. With improvement in the conception rate through A.I. and its doorstep delivery and propagation of pedigree breeding concepts, acceptability of A.I. is likely to increase rapidly.

2.2.2 Cryopreservation of Oocytes

In the last 10 years, considerable progress has been made with cryopreservation of oocytes. Viable oocytes have been recovered after freezing and thawing in various species of livestock. Birth of progeny from embryos produced from cryopreserved oocytes have been reported in cattle (Otai *et al.*, 1996; Ruane and Sonnino, 2006). The present efficiency and reliability of using frozen thawed oocyte for generating offspring is, however, still lower than with cryopreserved embryos.

2.3 Cryopreservation of Embryos

Embryo freezing is essential for operation of commercial ET programme. It facilitates use of surplus embryos and global movement of animal in the form of embryos.

Normally the medium that contains the embryos cools below its freezing point without ice crystal formation, a phenomenon referred to as super-cooling. Then, at some lower temperature, ice nucleation occurs, followed by a rapid rise in temperature due to the release of latent heat of fusion. To avoid extensive super-cooling, ice crystallization is induced in the extra cellular medium some 2°C below its freezing point (-4 to -7 °C) by seeding the medium with an ice crystal (Palasz and Mapletoft, 1996). Water in the cells of the embryo and between the ice crystals outside the embryos does not freeze at this temperature because of solutes lowering its freezing point. During further cooling and enlargement of ice crystals, the solute concentration rises and the embryo responds osmotically by losing water into the extra cellular unfrozen medium.

The required thawing rate depends on the freezing regimen used. When embryos are cooled slowly to temperatures between -27 and -40 °C and then rapidly to -196 °C (liquid nitrogen), thawing must be rapid e.g. about 200 °C/min. Cells treated in this way may contain some intracellular ice, and thawing has to be rapid to prevent injury from the recrystallization of that ice. On the other had, if embryos are cooled slowly to temperatures below -60 °C before transfer to liquid nitrogen, thawing is then normally done slowly at about 20 °C/min (Liebo and Mapletoft, 1998). Although both systems result in similar rates of embryos survival, more rapid techniques of freezing and thawing are preferred in the field. The freezing of bovine embryos is now commonplace and pregnancy rates are only slightly less than that achieved with fresh embryos (Liebo and Mapletoft, 1998).

Cryoprotectants such as glycerol in concentrations ranging from 1.0 to 2.0 M are required to ensure embryos survival after freezing. Recently, the use of highly permeating cryoprotectants such as ethylene glycol has allowed the direct transfer of bovine embryos without the necessity of microscope examination and cryoprotectants removal (Liebo and Mapletoft, 1998). With this approach, the embryo straw is thawed in a water-bath, much like semen, and the contents of the straw are deposited into the uterus of the recipient, much like AI. The direct transfer of 19,000 bovine embryos in Canada resulted in an overall pregnancy rate of 58% which was not different to that achieved by regular cryoprotectant dilution techniques. Several AI centers have now trained technicians to perform direct transfer and transfer of frozen/thawed bovine embryos is now becoming very similar to the use of frozen/thawed semen in AI.

However, freezing and thawing procedures are time consuming and require the use of biological freezers and a microscope. These steps can be replaced by a relatively simple procedure called virtification (Rall and Fahy, 1985). High concentrations of cryoprotectants are used and the embryos with cryoprotectants solution are placed directly into liquid nitrogen. Vitrification has much to offer in the cryopreservation of oocytes and IVF embryos, however, its greatest advantage is its simplicity in application. Vitrification procedures are now widely used experimentally and it is only a matter of time before they find commercial application. Recently, a procedure for the direct transfer of vitrified bovine embryos with pregnancy rates that did not differ from that of traditional techniques was reported (Van Wagtendonk *et al.*, 1996). Clearly, vitrification of bovine embryos in commercial bovine embryos transfer is on the horizon.

Freezing of embryos is an established commercial practice especially in cattle. In India, Misra *et*

al.(1992b) cryopreserved 700 cattle and 200 buffalo embryos and found almost similar (87 and 88%) post thaw embyo survival . However, post thaw survival rate was significantly higher when embryos were frozen at in early blastocyst/ blastocyst stage than morula. Over all pregnancy rates from frozen cattle and buffalo embryos were 31 and 20 %, respectively, which is low compared to 56.1% reported in USA (Hasler, 2001) and needs to be improved.

Embryos are normally stored in liquid nitrogen at -196°C. The only reactions that occur at -196°C are direct ionizations from background radiation. Consequently, storage times of more than 200 years are unlikely to produce any detectable reduction in survival or cause genetic change of frozen embryos.

2.4 Sexing of sperm

The technological advancement has reached to a stage where we can get the desired offsprings after insemination. Now a days sexing of semen is considered to be one of the most desirable reproductive technologies. Male is the first choice in meat industry as also in artificial insemination programme for production of quality semen. Semen can be sexed because X-sperm, which produce heifers, contain 3.8% more DNA than Y-sperm, which produce bull calves (Johnson and Welch, 1999). This technique, sorts X and Y chromosome bearing sperm with the help of a cell sorter/ cell flow cytometer, which has been patented (Johnson, 1992; Johnson and Welch, 1999) and is being used commercially (Siedel, 1999; Amann, 1999). With this technique, sex of calves can be predetermined with 90% accuracy (Siedel, 2003).

Another application is to obtain male calves from the very best cows in the herd to use them as breeding bulls. Sexed sperm could be especially useful for superovulation, in which case it often is desirable to obtain calves of one sex or another for a particular mating. One must be careful when using sexed semen for this application because more, rather than fewer, sperm typically are used for superovulated cattle, hence embryo production will be lower and more variable than with unsexed sperm (Schenk *et al.*, 2006). Nevertheless, the calves produced will be about 90% of the desired sex, so even if embryo production is reduced by one-third, this application may be appropriate (Seidel, 2007).

2.5 Embryo transfer technology (ETT)

Multiple ovulation and embryo transfer is a technology through which the reproductive potential of the female can be accelerated. The most important application of ETT is the production of AI bulls from the best-proven bulls and cows / buffaloes available. Importance of this technology can be gauged from the fact that in USA in 2003, embryo transfer produced 66 of the top 100 Total Production Index (TPI) international Holstein bulls.

In several countries around the world nucleus herds are now being developed and heifer offspring are being subjected to "Juvenile MOET" while male offsprings are being selected for the next generation of AI bulls (Smith and Ruane, 1987; Teepker and Keller, 1989) and genetic gains can be doubled. The intercontinental transport of a live animal may cost several thousands of dollars whereas an entire herd can be transported in the form of frozen embryos for less than the price of a single plane fare. However the reduced risk of infectious disease transmission is the overwhelming benefit for using embryos in international trade. This may be the single most important potential application of embryo transfer.

The commercial embryo transfer industry in North America developed with the introduction of continental breeds of cattle (Betteridge, 2003). The use of embryo transfer technology in cattle breeding has continued to increase (especially within the dairy industry) over the past 30 years with the movement toward genetic improvement as opposed to the production of desirable phenotypes (Smith, 1988). In Canada approximately 70% of the embryo transfer work is now being done on dairy cattle, and approximately 15,000 embryos are being frozen annually for export (Canadian Embryo Transfer Association Statistics, www.ceta.ca).

As per the IETS data retrieval committee report, a total of 670,711 in vivo-derived embryos (Table 1) and 291,845 in vitro-produced bovine embryos (Table 2), a total of almost 1 million cattle embryos were transferred in 2006. The distribution of in vivo-derived bovine embryos is worldwide and North America takes into account approximately 44% of the total ahead of Asia, South America and Europe and lastly Oceania and Africa. About 46 % of these embryos were transferred as frozen-thawed embryos. For in vitro-produced embryos, the vast majority of the embryos transferred come from South America (Brazil) and Asia (the People Republic of China, Japan and Korea). Embryo Transfer (almost exclusively in-vivo derived embryos) is also widely used in other ruminant species but with lower numbers than in cattle. In total, 43,000 sheep embryos, 24,000 goat embryos (mostly frozen-thawed) and 696 Cervid embryos have been reported. Equine and swine embryos were recorded with 15,695 equine embryos, almost all fresh, and 33,779 swine embryos, all transferred as fresh embryos. These figures are underestimated due to lack of national collectors in some countries or retrieval of partial data in a few others. However, they prove that the ET industry continues to grow and hence continues to bring benefits to the farming industry.

Table 1. Overall activity of in vivo derived bovine embryos in 2006

Continents	Flushes	Transferable embryos	Number of transferred embryos		
			Fresh	Frozen	Total
Africa	1,607	13,660	20,063	3,118	5,181 (0.8%)
N.America	64,711	415,596	131,510	161,095	292,605 (43.6%)
S. America	18,000	99,627	76,521	11,740	88,261 (13.2%)
Asia	18,919	139,534	60,730	123,195	183,925 (27.4%)
Europe	15,859	94,090	36,033	51,808	87,841(13.1%)
Oceania	2,816	15,240	7,849	5,049	12,898 (1.9%)
Total	121,912	777,747	314,706	356,005	670,711

Table 2. The number of bovine in vitro produced embryos transferred in 2006

Regions	Transferable embryos	Number of transferred embryos		
		Fresh	Frozen	Total
Africa	-	-	-	-
N. America	134,162	4,306	3	4,309(1.5%)
S. America	204,469	196,759	32	196,791(67.4%)
Asia	86945	20,859	61,448	82,307(28.2%)
Europe	13,942	2,763	4,082	6,845(2.4%)
Oceania	1,846	1,390	203	1,593(0.5%)
Total	441,364	226,077	65,768	291,845

Pregnancy rate following transfer of fresh and frozen embryo in cattle is reported to be 68.3 to 77.1% and 56.1% (Hasler, 2001), respectively. In India, pregnancy following ET was reported to be highly variable (generally 20 to 50%), however, occasionally higher conception rates (72.72%, Siddiqui *et al.*, 2001; 78.6%, Misra *et al.*, 1992a) had also been reported. In buffalo, initially, the conception rate following ET was reported to be only 10-18% (Alexiev *et al.*, 1988; Kurup, 1988), however, subsequently, following transfer of mostly non freezable embryos (grade II & III), the pregnancy rate improved significantly to 25 -26.4 % on farm (Misra *et al.*, 1992b, 1999b) and 29.4% in the field (Misra *et al.*, 1999a). A higher conception rate of 60% was also observed following transfer of only Grade I embryos (Misra *et al.*, 1999b).

Animal Cloning

In animals, "Cloning" is typically the production of genetically identical individuals. Cloning occurs naturally in many plants and microorganisms, and in some lower animals. An animal clone can be produced either by embryo splitting (as occurs in nature)/ transfer of embryonic blastomeres or somatic cells as donor nuclei. The advantage of cloning somatic over embryonic tissue is that the former provides the opportunity to first evaluate the merits of the animals to provide the cells used for cloning. Somatic cell nuclear transfer (SCNT) is a powerful technique and potentially it could be used for:

a. Multiplication of desired animals

b. Minimizing the genetic variation in the experimental animals

c. Conservation of elite and rare animals

d. Production of genetically modified farm animals to make modification of milk, growth, disease resistance, xenotransplantation etc.

e. Production of stem cells and their therapy.

Cloning technique could be a competitive technology. India has the large population of crossbreds and crossbreeding of non descript and low producing cattle stock (*Bos indicus*) with exotic high milk producing *Bos taurus* breeds is the national breeding policy, however, about 25% heterosis (Cunningham and Syrstad, 1987) achieved in the first cross (F1) is difficult to maintain in the subsequent crosses and methods for retention of heterosis are generally too complex for implementation. Alternately, embryos could be produced by using *Bos indicus* oocytes and *Bos taurus* semen *in vivo* or in-vitro or it might be possible in future to use cloned embryos of outstanding F1 individuals. Besides production of multiple copies of the proven/ elite animals (reproductive cloning), use of embryos produced by SCNT for the production of embryonic stem cells and their subsequent use for cell/ gene therapy (therapeutic cloning) is the biggest application of this technology.

Transgenesis

Transgenesis (or gene transfer) is defined as insertion of forigen DNA and its stable integration into host genome in such a way that it functions in the receiving species and is passed on from one generation to the next. By using conventional methods, several generations are required to fix genetic changes within a population. Transgenesis is considered as feasible and rapid method for direct genetic manipulation of the animals. A number of methods have been used to produce

transgenic animals such as DNA transfer by retroviruses, microinjection of genes into pronuclei of fertilized ova, injection of embryonic stem (ES) cells and/or embryonic germ (EG) cells previously exposed to foreign DNA into the cavity of blastocysts, sperm-mediated exogenous DNA transfer during *in vitro* fertilization, liposome mediated DNA transfer into cells and embryos, electroporation of DNA into sperm, ova or embryos, nuclear transfer (NT) with transfected somatic cells, ES or EG cells (Neimann and Kues, 2003). However, the main methods used for the creation of transgenic animals are pronuclear microinjection, somatic cell cloning and embryonic stem cell-mediated gene transfer (Clark and Whitelaw, 2003).

Gene transfer in animals has been used for modifying the fat or protein synthesis in the mammary glands and numerous proteins have been produced in the mammary gland of transgenic sheep, goat, cattle, pig and rabbit (Kues and Niemann, 2004). Transfer of growth hormone gene in pigs, transferring cysteine synthesis gene into sheep for enhanced wool production (Powell *et al.* 1994), imparting resistance to influenza virus in pig (Muller *et al.*, 1992), human ⇨ anti-trypsin in sheep (Carver *et* al.,1993), ⇨-lactalbumin in cow (Colmon, 1996), tissue plasminogen activator and antithrombin III (AT III) production from goat which are at phase III trial (for review see Niemann and Kues, 2007) and cloning of the cow from transgenic donor cells that express a Lysostaphin gene, rendering the cow resistant to mastitis have been reported (Wall *et al.*, 2005).

Stem cell technology:

Stem cells are unspecialized cells that have ability to self-replicate for indefinite period and differentiate into other cell types under certain physiological or experimental conditions. They can be induced to become cells with specific functions like beating cells of heart muscle or insulin producing cells of pancreas, blood cells, neural cells etc., which can be utilized for the treatment of many chronic diseases which are considered incurable until now. Stem cells can be obtained from an embryo or an individual.

Embryonic Stem Cells:

Embryonic stem (ES) cells are obtained from the inner cell mass of the early embryo. If these cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously and can form muscle, nerve and many other cell types like heart muscle cells, blood cells etc. ES cells derived transgenic animals can be commercially used for the biomedical industry, xenotransplantation, production of pharmaceutical proteins in the milk of animals etc.

Studies on mice ES cells which converted into oocyte (Hubner *et al.*, 2003) indicate that stem cell lines can be generated in cattle from genetically superior females to allow an unlimited number of oocytes to be derived. In pig, ES cells differentiated into cardiac-like muscle cells may help in improving cardiac function in a swine myocardial infarction (Moscoso *et al.*, 2005). Others have also reported generation of bovine pluripotent ES–like cells from IVF or NT embryos (Mitalipova *et al.*, 2001; Saito *et al.*, 2003). It has also been suggested that bovine ES cells may serve as a better model than mouse ES cells for human ES cell tissue regeneration studies. (Wang *et al.*, 2005).

Somatic Stem Cells

These are unspecialized cells found among specialized cells in a tissue or organ, which can renew itself and can differentiate to yield the major specialized cell types of the tissue or organs.

Unlike embryonic stem cells, which are defined by their origin, the origin of the somatic cells in mature tissues is unknown. These adult stem cells seems to have the ability to differentiate into a number of different cell types (Haematopoietic stem cells- RBC, B-lymphocytes, T-lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, macrophages and platelets; stromal cells (mesenchymal stem cells) which can give rise to osteocytes (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells) and cells of tendons and if this differentiation can be controlled in the laboratory, these cells may be used for the therapy of several diseases.

Other Emerging Reproductive Technologies

Transplantation of ovarian tissue and germ cells {e.g. spermatogonial stem cells (SSC)} are emerging technologies with potential for future use. Successful transplantation of ovarian tissue has been reported in rodents, sheep, mammoset, monkeys and human (Donnez *et al.*, 2004). Successful whole sheep ovary crypreservation and autotransplantation has recently been reported (Revel *et al.*, 2004). Germ cells transplantation research has been developed as a unique approach for the study of gametogenesis and germ line manipulation. To date, successful germ cell transplantation has been reported in several livestock species eg. Transplantation of SSC in cattle (Izadyar *et al.*, 2003) and goats (Honaramooz *et al.*, 2003). Gamete intra-fallopian tube Transfer (GIFT), an assisted reproductive technology has been used to produce pregnancies in mares (Carnevale, 1996; Hinrichs *et al.*, 1998). Intra-cytoplasmic sperm injection (ICSI), has been successfully used cattle (Goto *et a.*, 1991), mares (Squires *et al.*, 1996; Cochran *et al.*, 1998), dogs and pig (Kim *et al.*, 1998).

Conclusion:

- There is urgent need to increase livestock output, especially in developing countries to meet the growing demand. Which is possible only by improved efficiency rather than increasing the number of animals? Available biotechnologies can help in achieving this goal.
- AI will continue to be the most important reproductive biotechnology to act as the vehicle for the wider dissemination of elite germ plasm, as semen. There is need, however, especially in India, to improve the quality of AI services to increase the conception rate following AI and then progressively more and more breedable cows and buffaloes be brought under AI coverage.
- The bulls used for the semen collection must be produced from the elitist parents using ETT and genetically evaluated. Sibling testing using ETT (open nucleus breeding system) and MAS of bull calves soon after birth may prove useful in improving the gains through AI.
- The large-scale application of embryo technologies for enhancing reproductive function and genetic improvement is to be taken commercially. The sperm sexing and OPU–IVEP technology have the potential to produce embryos and the progeny of the desired sex at a much lower cost.
- Somatic cell cloning by nuclear transfer is a relatively new technology with many potential applications.
- The technology of gene transfer has been used successfully to produce pharmaceutical products in animal tissues and animals with increased resistance for certain diseases.

This however, needs to be researched further to be of value for improving normal animal health, productivity and xenotransplantation.

- Use of embryonic stem (ES) cell lines for the in-vitro production of oocytes and sperm will facilitate availability of unlimited number of gametes of genetically superior males and females to improve livestock production and reproduction.
- Reproductive biotechnology, however, possess enormous potential for improving the livestock productivity and animal genetic resources conservation, however, the adoption of reproductive biotechnology for livestock production is governed by economic factors. For these reasons, most developments in reproductive biotechnology take place in the developed countries, and acceptance of these technologies in developing countries is poor.

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POLYMORPHISM OF TOLL-LIKE RECEPTORS AND THEIR ROLE IN IMMUNITY

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Being a critical first line of defense and evolutionarily conserved, the innate immune response plays important role to fight bacterial, viral and fungal pathogens, without requiring any prior exposure. Adaptive immunity induces acquired resistance against microorganisms through random somatic rearrangements of genes encoding immunoglobulins and T cell receptors, thus generating a high level of diversity of receptors in response to microbial invasion. Acquired resistance is not vertically transmitted and reflects the infectious history of every individual. In contrast, innate immunity relies on recognition of antigens by a small number of weakly specific receptors designated Pattern-Recognition Receptors (PRR) and is vertically transmitted by germinal cells. The PRR are expressed on macrophages dendritic cells and B-lymphocytes and recognize antigenic structures highly conserved in the living world, termed Pathogen-Associated Molecular Patterns (PAMP). Well conserved features in pathogens like bacterial cell-surface lipopolysaccharides (LPS), lipoproteins, lipopeptides and lipoarabinomannan; proteins such as flagellin from bacterial flagella and double-stranded RNA of viruses or the unmethylated CpG islands of bacterial and viral DNA; are specifically recognized by different types of TLRs.

PRR are secreted (complement, lectins), or expressed at the cell surface to induce endocytosis or signaling (Toll-like receptors or TLRs). Toll-like receptors (TLRs) play a central role to mount innate immune response through recognition of conserved pathogen associated molecular patterns to initiate an adaptive immune response through signaling pathways. Toll is a protein originally discovered in *Drosophila* in 1985, governing dorsal-ventral polarity and also required to mount an immune response against fungal infection. Mammals are considered to possess 10-13 TLRs recognizing different ligands of various pathogens including parasites at present.

Mammalian TLRs have been functionally characterized and are classified mainly on the basis of their stimulation by different ligands. Deficiency in single TLR rarely shows the extreme susceptibility to infections leading to functional significance of TLR diversification in vertebrates in contrast to *Drosophila*. TLRs induce the inflammatory response against microorganisms through NF-kB, a cytoplasmic factor controlling transcription of many genes, including cytokines (TNF, INF, IL-1, IL-2, IL-8, IL-12.) and defensins. So, within few minutes following microbial aggression, the inflammatory response is rapidly triggered to destroy infectious agents and to generate a long-term memory against pathogens.

TLRs and adaptive immune response

Engagement of TLRs with their ligands leads to the production of various proinflammatory cytokines, chemokines and effector molecules, depending on the cell type. Recently, TLRs were observed to influence the development of adaptive immune responses, presumably by activating antigen-presenting cells. This has important implications for our understanding of how the host triggers its immune response as a function of specific pathogen recognition. Stimulation of TLRs by PAMPs or endogenous ligands leads to intracellular signaling via interaction of the cytoplasmic TIR domain with the adaptor protein MyD88, which appears to function with all TLRs. Signals are then propagated through a cascading series of interactions with downstream cytoplasmic proteins, ultimately leading

Table: TLR ligands, their location and expression in cell types

Receptor	Ligand(s)	Ligand location	Location	Cell types
TLR 1	Multiple triacyl lipopeptides	Bacteria	Cell surface	· Monocytes/macrophages A subset of dendritic cells B lymphocytes
TLR 2	Multiple glycolipids multiple lipopeptides lipoteichoic acid peptidoglycan, HSP70 zymosan, Numerous others	Gram positive bacteria Host cells Fungi	Cell surface	· Monocytes/macrophages Myeloid dendritic cellsMast cells
TLR 3	Double-stranded RNA, poly I:C	Viruses	Cell compartment	· Dendritic cells B lymphocytes
TLR 4 lium	Lipopolysaccharide several heat shock proteins, fibrinogen fragments hyaluronic acid fragments Numerous others	Gram-negative bacteria and host cells heparan sulfate	Cell surface	· Monocytes/macrophages Myeloid dendritic cells Mast cells Intestinal epithe-
TLR 5	Flagellin	Bacteria	Cell surface	· Monocyte/macrophages · A subset of dendritic cells · Intestinal epithelium
TLR 6	Multiple diacyl	Mycoplasma lipopeptides	Cell surface	· Monocytes/macrophages · Mast cells · B lymphocytes
TLR 7	Imidazoquinoline loxoribine (a guanosine analogue), bropirimine single-stranded RNA	Small synthetic compounds	Cell compartment	· Monocytes/macrophages Plasmacytoid dendritic cellB lymphocytes
TLR 8	Small synthetic compounds; single-stranded RNA		Cell compartment	· monocytes/macrophages · a subset of dendritic cells · Mast cells
TLR 9	Unmethylated CpG DNA	Bacteria	Cell compartment	· Monocytes/macrophages · Plasmacytoid dendritic cells · B lymphocytes
TLR 10	Unknown	Unknown	Cell surface	· Monocytes/macrophages · B lymphocytes
TLR 11	Profilin	Uropathogenic	Cell surface bacteria	· Monocytes/macrophages · Liver cells · Kidney · Bladder epithelium
TLR 12	Unknown		?	
TLR 13	Unknown		?	

to the activation of NF-kB and initiation of the adaptive immune response. Most studies have focused on the role of TLRs and components of their signaling pathways in the control of Th1-type immune responses, and on the implications for their use as antimicrobial agents, such as adjuvants in vaccines, or to treat or prevent the Th2-type dominated immune responses seen in allergies. TLR-deficient mice have been described and used to come to these conclusions. Although controversial, there is also evidence that TLRs may be important for Th2-type responses, possibly by augmenting the overall maturity of dendritic cells.

Therapeutic implications of TLRs

The development of therapeutic strategies that harness the power of the innate immune system could lead to novel treatments for several diseases that are difficult to treat using conventional approaches. Because of their ability to modulate adaptive immunity, Toll-like receptors represent strategic therapeutic targets for diseases that involve inappropriate adaptive immune responses, such as sepsis, autoimmune disorders, cancer and allergy. Multiple TLR-ligand interactions are required to induce effective host resistance to pathogens, which has important implications for designing improved strategies for vaccination and immunotherapy against infectious diseases. Individual TLR7, TLR8 and TLR9 agonists have been used successfully as adjuvants to boost $CD4^+$ and $CD8^+$ T-cell response to microbial vaccines. In some diseases such as sepsis and several autoimmune disorders, TLR signaling has been found to be an integral component of disease progression. For such diseases, therapeutic approaches aimed at reducing the level of TLR signaling are being explored. In other diseases such as cancer and allergy, where ineffective or inappropriate immune responses are partially to blame for disease progression, therapeutic strategies are being designed to take advantage of the fact that TLR signaling is able to shift the immune system towards an inflammatory TH1 mode, a transition which can, for example, lead to tumour eradication or an amelioration of allergy symptoms. Because TLR stimulation leads to strong protective T_H1 immune response, CpG oligo-deoxy-ribonucleotides are being investigated as a potential adjuvant, not only to enhance the effectiveness of vaccination but also to enhance overall immune readiness before pathogen exposure.

Polymorphism in TLRs

Recent TLR studies have demonstrated that the mammalian innate immune system possesses a greater degree of specificity than was previously hypothesized, with a highly developed ability to discriminate between self and foreign pathogens. This scientific progress has remodeled previous ideas about the diagnosis and treatment of infectious, immune, and allergic diseases. Moreover, a recent study illustrates the importance of defining TLR polymorphisms by demonstrating that several human TLR5 mis-sense mutations abolish bacterial-induced signal transduction. Nevertheless, the application of this knowledge to domestic food-animal species and their respective populations remains somewhat limited. Genomic organization of livestock TLR genes may promote our understanding of their evolution and help in the identification of genes underlying disease resistance traits.

Swine TLRs are predicted to be associated with responses to infectious diseases such as pneumonia. In a study single nucleotide polymorphisms (SNPs) in the coding sequences of porcine *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* genes in 96 pigs from 11 breeds elucidated 21, 11, 7, 13, and 11 SNPs, respectively, which caused amino acid substitutions in the respective TLRs. Distribution of these non-synonymous SNPs was in the leucine-rich repeats, particularly in *TLR1*. Heterogeneity

of *TLR* genes was preserved in various porcine breeds despite intensive breeding that was carried out for livestock improvement suggesting that the heterogeneity in *TLR* genes is advantageous in increasing the possibility of survival in porcine populations. In sheep possible correlation between TLR1, 2 & 4 gene mutations and the susceptibility of the udder to bacterial infections and also the associated breed-dependent aspects of somatic cell count has also been established.

In bovine, radiation hybrid mapping of ten TLRs (TLR1to TLR10) has been carried out and these ten TLRs have been mapped to seven chromosomes (BTA6, 8, 16, 17, 22, 27 and BTAX). Transcription profiling of the three TLR genes (TLR1, 6 & 10) in different bovine tissues showed similar expression profiles for TLR1 and TLR6, which indicate a co-regulation of these two genes in cattle. TLR10 had a different expression profile, pointing toward a stronger functional diversification compared to TLR1 and TLR6.

Although SNPs detected in different coding and non-coding regions of various TLRs have been associated with susceptibility to many infectious and non-infectious diseases in human beings, the work is being initiated in livestock species as well now. By screening of different breeds of *Bos indicus* and *Bos taurus*, 32 SNPs resulting in 20 haplotypes have been detected with in TLR4 of bovine. Polymorphisms in the genomic sequences of bovine TLRs 3, 7, 8, and 9 has been analysed in nine breeds of *Bos taurus* and *Bos indicus* and comparative sequence analysis revealed a total of 139 polymorphisms, which include single-nucleotide polymorphisms and insertion–deletion polymorphisms. Whereas, within bovine TLRs 1, 5, and 10 comparative sequence analysis for 10 bovine breeds derived from *Bos taurus* and *Bos indicus* revealed 98 polymorphisms (92 SNPs and 6 indels), with at least 14 nonsynonymous SNPs located within predicted TLR domains considered to be of functional significance. These studies reveal presence of considerable variation within bovine TLRs and the same could be studied for their association with disease resistance in our indigenous cattle and buffalo populations. We have attempted and found association of one SNP in intron1 of TLR4 gene to be having significant effect on susceptibility to *Pinkeye* in American Black Angus cattle recently (Unpublished report).

Conclusion

The discovery of the Toll-like receptors finally identified the innate immune receptors that are responsible for many of the innate immune functions that had been studied for many years. Interestingly, TLRs seem only to be involved in the cytokine production and cellular activation in response to microbes. A better understanding of the processes by which TLRs regulate adaptive immune response may help not only developing improved ways to treat infectious diseases but also new approaches to prevent and treat the allergic and autoimmune disorders as well.

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SESSION-IX

Poster Session

Chairman : **Dr. M.V. Subba Rao**

Co-Chairman : **Dr. V. Ramaswamy**

Rapporteur : **Dr. Anil Kumar**

ABSTRACTS

9.01 PCR amplification of a potent T- cell inducer antigenic protein of *Mycobacterium avium* subspecies paratuberculosis
Rajib Deb, P.P. Goswami, S. Chandrasekhar, Ananta Kr. Das & N.S. Prasad

9.02 Effect of heavy metal pollution of water on response of fish lymphocytes to mitogenic stimulation
Madhu Saxena, H M Saxena, Paviter Kaur & Kamaldeep Kaur

9.03 Comparison of serum and whole blood PCR for diagnosis of goat brucellosis
Jyoti Vohra, Ranjeeta Kumari, V. K. Gupta and V. S. Vihan

9.04 Salmonellosis in Human and Animal
Shweta Singh, R. K. Agarwal, Kuldeep Kumar & Lakshyaveer Singh

9.05 New Strategies for Improving Viral & Cancer Vaccines
Bhaskar Ganguly, Sudhir Kumar, Umapathi V., Tanuj Ambwani

9.06 Nano- drug delivery
Kamal Tewari, Bhaskar Ganguly

9.07 Hydrogels for Nano Drug Delivery: A Review
Sanjay Basera, Kamal Tewari, Anoop Nautiyal

9.08 Pant derived edible vaccines
Devki Pilkhwal

9.09 Micro and Nano Drug Delivery System in Cancer Therapy
Sanjay Basera, Kamal Pant, Anoop Nautiyal

9.10 Polymeric Nano Particles in Vaccine Technology and Vaccine Delivery : A Review
Jitendra Kumar Verma; Rathish. R. L

9.11 Microbial L-Asparginase: a potent anti tumour enzyme
Deepak Singh Bisht

9.12 Recent Approaches in Embryo Transfer Technology
Aman Kumar, Amit Pal Panwar

9.13 Nanotechnology
Abhishek Kumar

9.14 Phage Therapy: A Viable Alternative To Antibiotics
Amit Pal Panwar, Aman Kumar

9.15 RNAi – Breakthrough In Molecular Medicine
Aman Kumar, Amit Pal Panwar

9.16 Effect of Chemical Industry Effluent on Lymphocyte Transformation Responses in Mice
Seema Agarwal, D.K.Agrawal, Virendra Garg & Yogesh Upadhyay

9.17 Effect of Paper and Pulp Industry Effluent on humoral immune response in mice measured by ELISA
Yogesh Upadhayay , D.K. Agrawal, Seema Agrawal & Munish Batra

9.18 Essential oil composition from aerial parts and antibacterial activity of *Bupleurum candolii*
Rajesh K. Joshi, Mrigendra S. Rajput, Jugendra Pal

9.19 Isolation, Identification, Antibiogram of *Campylobacter jejuni* in Broilers from retail outlets
Vishal Kumar Sharma, Sri Krishna Mishra, Rohit Kumar Mishra, M. Jeyakumar, Sanjeev Kumar, S. Malmarugan & N. Dorairajan

9.20 Apoptosis in chicken embryo fibroblast culture induced by infectious bursal disease virus
Amit Panwar, Umapathi V, Tanuj Ambwani, B.D. Lakhchaura

9.21 Documentation of Indigenous Knowledge/ Practices Related to Mastitis and Swelling on Udder in Uttarakhand State
Balvir Singh, A. K. Ghosh and Mohammad Shifa Tasal

9.22 Novel Vaccine Delivery Technology
Abhishek Pandey

9.23 Application of Bioinformatics in Molecular medicine
Abhishek Pandey

9.24 Analysis of Proteins of Fowl-Pox Virus by SDS-PAGE
Madanpal, Rahul Sati, Ranum Dabas and V.D.P. Rao

9.25 Propagation and Adaptation of Fowl-Pox Virus In BHK-21 Cell Line
Ranum Dabas, Rahul Sati, Madanpal, and V.D.P. Rao

9.26 Serological Detection of Fowlpoxvirus in Infected Chorio-Allantoic Membrane and CEF Cell Culture
Rahul Sati, Ranum Dabas, Madanpal and V.D.P. Rao

9.27 Evaluation of pesticide induced hepatotoxicity in hepatocyte culture
Pandey. S. K., Singh, S. P., Mehta, G. and Ambwani, T.

9.28 Effect of Nitric oxide upon in vitro replication kinetics of Infectious Bursal Disease virus
Sarita Jena, Umapathi V., Sudhir Kumar, Tanuj Ambwani & Mumtesh Kumar

9.29 AP-PCR Analysis in Poultry *Salmonella* Isolates
R. A. Siddique, Mumtesh Saxena, Tanuj Ambwani and B.D. Lakhchaura

9.30 Colloidal gold based Immunochromatographic Assays *Salmonella* Isolates
Shalini Bhutani, Priyamvada Kumari, Sonu Ambwani and Anil Kumar

9.31 Immunosensors
Shalini Bhutani, Priyamvada Kumari, Sonu Ambwani and Anil Kumar

9.32 Antibody Engineering: a breakthrough in Immunotechnology
Abay Gupta, Awadesh Kumar, Sonu Ambwani, Tanuj Ambwani and Rajesh Chandra

SESSION-X

Plenary Session

Chairman	:	**Dr. P. Thangaraj**
Co-Chairman	:	**Dr. K.S. Palaniswamy**
Rapporteur	:	**Dr. A. Thangavel**

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With best compliments from--

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Our Mission is to

"Adopt, Introduce, Integrate, Promote, Manage, Organize & undertake all activities viably for the genetic improvement & subsequent increase in productivity of Cattle & Buffaloes in the State".

Our Objectives are:

- To carry out cattle and buffalo breeding activities in accordance with the states breeding policy under one common umbrella.
- Progressively bring all breedable cattle and buffalo population under the gambit of organized artificial insemination Program.
- Strengthening of the natural breeding infrastructure for cattle and buffalo in the remote corners of Uttaranchal.

Livestock Insurance Scheme: As the livestock keeping is one of the major resource for the livelihood of rural households of our country & unfortunate calamities causing death of livestock is resulting in even worsen condition of the poor households keeping livestock. To overcome this constraint of losses of livestock the Government of India has launched Livestock Insurance Scheme (LIS) in 100 districts across the country on pilot basis. In the state of Uttarakhand Haridwar & Udham Singh Nagar districts are selected for the implementation of LIS on pilot basis. The ULDB is State Implementing Agency for the implementation of this Scheme. This scheme is being implemented on 50% cost sharing basis for the insurance of elite milch cows & buffaloes for 01 to 03 year duration & a maximum of 02 elite milch cows & buffaloes of one household can be covered under this scheme. The LIS is implemented in the state with active support from dept. of Animal Husbandry & Dairy Development Department & till now a total of 1490 elite milch cow & buffalo are being insured under this scheme.

Other Activities & Achievements:

- **Artificial Insemination Program: Presently the** board is supplying Deep Frozen Semen, Liquid Nitrogen & other breeding inputs to 854 Artificial Insemination Centers on door delivery basis.
- **Natural Breeding Program:** The board has procured & distributed 1197 elite cow & buffalo breeding bulls on custodian basis for the natural breeding program in the remote hilly areas of the state.
- **Training Program:** The board has provided A.I., breeding & livestock management related trainings to 11756 trainees (Veterinary Officers/ Livestock Extension Officers/ Pharmacists/ Farmers) at esteemed organizations & ULDB Training Centers located at Pashulok, Rishikesh & Animal Breding Farm, Kalsi.
- **Deep Frozen Semen Production Program:** The board has an ISO 9001:2000 certified Deep Frozen Semen Production Center (DFSPC) at Shyampur, Rishikesh. The DFSPC is maintaining elite bulls of H.F., Jersey, their crosses, Red Sindhi, Sahiwal & Murrah. **The DFSPC has also received "Certificate of Merit" from hon'ble Minister of Agriculture, Government of India in the year 2005-06.**
- **Embryo Transfer Program:** The board by its State Embryo Transfer Center, Lalkuan, Nainital has able to produce **183** progenies by adopting this modern technique at the farmer's door step.
- **Animal Breeding Farm, Kalsi:** With the view of conserving Red Sindhi breed of native cattle the board has established one state of art Embryo Transfer Laboratory at Animal Breeding Farm, Kalsi.
- **Field Performance Recording Program:** For identifying elite milch animals the board has initiated Field Performance Recording Program. At present a total of 1681 elite milch animals are under milk recording program.
- **Fodder Development & Animal Nutrition Project: Looking to the scarcity of fodder the board has initiated projects for "Establishment of Center of Excellence of Fodder Grasses" at 16 Van Panchayats & 13 Government Farms and "Grass Land Development Program" at 09 Community Lands located through out the state of Uttarakhand. The board is also implementing Pasture Land Development; Certified Seed Production; Fodder Grasses & Fodder Trees Development programs in the Van Panchyat & also producing & distributing of Compact Feed Supplement Blocks to the remote hilly area of state of Uttarakhand.**


COLLEGE OF VETERINARY SCIENCES