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Role of Immunology in Veterinary Profession

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Although the way ahead for immunology is full of pitfalls and difficulties, this is indeed an exhilarating prospect. There is no danger of a shortage of forthcoming excitement in the subject. Yet, as always, the highlights of tomorrow are the unpredictabilities of today- Cesar Milstein

Immunology is the study of the immune system in health and disease. Immunology was born of Microbiology following dramatic success of Louis Pasteur's germ theory of disease and Robert Koch's famous Koch postulates for proof of etiology. However, it was only in the 20th century that a comprehensive understanding was obtained on the development, mode of action and interaction of antibodies and antigen-specific lymphocytes, which form the two major arms of the immune system. Immunology has a rich history of transforming discoveries and continues to improve public health through new advancements. The eternal appeal of immunology lies in the fact that it is highly dynamic and provides insights into every aspect of biological and biomedical research. Modern day immunology has become increasingly specific and state-of-the art in its manipulation of the body's immune system through vaccines, drugs, immunotherapy, monoclonal antibodies etc. to achieve a desired therapeutic or prophylactic effect.

Veterinary immunology is dedicated to the improvement of animal health and production. Animals, be it wild, domestic, farm, companion, aquatic or marine animals suffer from diseases when they are exposed to infectious agents or when their immune system does not function properly or become hyperactive. These animal infections can have extensive effects on human working sectors, like food, agriculture, and animal husbandry and can deeply affect public health and ecosystem health. It is of utmost importance that animal diseases are effectively treated and controlled to improve food safety, public health, and overall animal and human welfare. Therefore, here comes the indispensable role of veterinarians.

There is substantial contribution of veterinary research to the advancement of human medicine. From Pasteur's experiments on rabies and anthrax vaccination in sheep and Koch's studies on tuberculosis to Ralph Brinster's pioneering work in embryo transplantation and Peter Doherty's work on major histocompatibility complex proteins, the knowledge of comparative or veterinary research formed the basis for many contemporary discoveries in medical sciences. Moreover, research using small and large animal models has been an important component of almost every advancement in immunology in the recent decades. For example, the development of vaccines, antibiotics or drugs has relied on research in animal models, the use of insulin to treat diabetes or antiretroviral drugs against HIV, and research endeavors in cancer chemotherapy and immunotherapy would not have been possible without the use of animal models. The use of small and large animal models remains crucial for scientific breakthroughs in the future. To this end, immunological studies on well-controlled experimental models using rodents and small and large domestic animals and on emerging and reemerging infectious diseases, especially zoonotic diseases, provide valuable means of manipulating immune system and of evaluating novel therapeutics and prophylactics. Therefore, understanding the relationship between the immune system of animals and the diagnosis, treatment and prevention of disease is critical in being a learned veterinarian.

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Immunologists working on the immune system of laboratory, domestic, and wild animals pursue both fundamental and applied research objectives. A basic understanding of the immune system of animals is important to identify the factors, which either lead to or prevent onset of clinical disease and damaging outcomes. Moreover, domestic animals represent the greatest diversity in innate and adaptive immunity to counter disease outcomes in all of medicine. The applied research objectives relate to the immunological improvement of animals' resistance to diseases and include studies on disease pathogenesis (e.g. hypersensitivity, autoimmunity, immunotolerance), immunodiagnostics (monoclonal antibodies, ELISA, serology), treatment (drugs, antibiotics, pharmaceuticals), vaccination (antigens, adjuvants, delivery systems, vectors), genetic selection (biomarkers of disease resistance, welfare and stress), chemotherapy (inhibitors, antimetabolites, antibiotics), and immunotherapy (cytokines, chemokines, immunosuppressive agents).Large animals have advantages for specific immunological studies such as lymphocyte trafficking and homing in *in vivo* cannulation models, fetal-maternal immunity (gnotobiotic pigs), transplantation immunology (pig xenografts in man), and models for infectious disease (non-human primates for tuberculosis, HIV, Hepatitis), genetic disorders (non-human primates for Diabetes, Parkinson's and Alzheimer's disease) and cancer (dog and pig).Therefore, veterinary clinicians should understand the immunological features and differences between breeds, strains and species, which will help them to approach proper treatment and care of animals. For instance, to adopt suitable immunotherapy in autoimmune diseases or chemotherapy in cancers in companion animals or to evaluate herd immunity after a mass vaccination program or to use serodiagnostic tests for routine disease diagnosis or epidemiological studies.

Vaccinology is concerned with the development of vaccines and preceded immunology in history when Edward Jenner demonstrated protective effect of cowpox against smallpox in 1798. Vaccines are recognized as the most important medical intervention that has saved millions of lives. Although many of the earlier vaccines have been developed empirically but a fundamental understanding of infectious disease immunology has led to the development of novel human and veterinary vaccines. Investigations of the function of the immune system in animals, host and pathogen genomics, and vaccination of long-lived animal species (dog, cattle, horse) offers unique promises for development and efficacy testing of novel vaccines.

One health initiative has been gaining momentum over the last decades and promotes cross-sectoral and interdisciplinary engagement in all health matters. Novel approaches for human, animal, and environmental health are expeditiously required in the wake of global rise in infectious disease outbreaks (Zika, Ebola), reemerging infectious diseases of humans and animals (HIV, avian influenza, Dengue, SARS, Ebola), non-communicable diseases (diabetes, cancer), antibiotic resistance (MRSA, VRE, MDR-TB), and lifestyle diseases (obesity, allergy, air-pollution induced lung diseases). The field of immunology encompassing many scientific disciplines in both human and veterinary medicine is one of the core areas of focus and research in one health initiative where veterinary immunologists have to play a greater role in protecting animal, human and ecosystems' health.

Immunologists are in a unique position to address health challenges in human and veterinary medicine and production challenges in animals. Increased investment and resource mobilization in immunology is required to facilitate advances in animal health, diagnosis and prevention of diseases and increased animal production for higher quality and safer foods. In Indian context, there should be better funding and infrastructure for immunology research in animals, improved interactions between veterinary immunologists and veterinary clinicians, a greater medical-veterinary immunology network, intercalated programs, summer school, or internships and possibly co-location of veterinary and medical schools.Future immunology research will continue to explore the complexities of the immune system through increasing multi-disciplinary research where veterinarians trained in immunology will have an important role to drive immunology forward to find new ways of improving human and animal health.

Immunogenicity Testing in the Era of Reverse and Systems Vaccinology

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ABSTRACT

Immunogenicity testing is an essential step in the preclinical phase of vaccine development and the quality control of commercial vaccines. This often employs protection of the host against pathogen challenge. Alternatively, *ex vivo* or *in vitro* measurement of immune parameters that correlate with protection may be done. Immune markers of infection, disease and vaccine-induced protection are being searched by employing modern approaches, including immunomics, computational immunology and systems vaccinology. Various humoral and cellular immune parameters are suggested as potential correlates or surrogates of protection induced by human and animal vaccines. *Ex vivo* and *in vitro* assays that measure immune cell proliferation, phenotypes, signaling, cytotoxicity, production of cytokines, antibodies, etc. provide an early and/or ethically improved readout of vaccine immunogenicity, thereby saving costs, time and animal suffering. Whole blood, peripheral blood mononuclear cells (PBMCs) or monocyte-derived dendritic cells (MoDCs)-co-cultured with PBMCs or T cell subsets can be used in these assays to examine primary or recall immune responses. In vitro antigen-primed MoDC: T cell co-culture models and artificial immune systems are being standardized and validated for evaluation of vaccine immunogens. The usefulness of immunogenicity testing 'outside animals' is likely to increase in future, with its focus on measuring the correlates of protection or surrogates of B and T cell immunity, which are being established for increasing number of current and future vaccines in the era of 'reverse' and 'systems' vaccinology.

Keywords: Immunogenicity testing, *ex vivo/in vivo* methods, rational vaccine discovery, vaccine efficacy evaluation

INTRODUCTION

Vertebrates possess innate and adaptive arms of immune system to respond to a diverse array of foreign substances, called molecular patterns and antigens (Ags), respectively. Generation of both Ag-specific B and T cell immune responses involves complex molecular and cellular interactions in sequential cognitive, proliferative and effector phases (Moser & Leo, 2010; Abbas et al., 2018). Proteins represent a very large class of dominant Ags, which harbor B epitopes and T epitopes bound specifically by B and T lymphocyte clones, respectively (Sanchez-Trincado et al., 2017). 'Immunogenicity' is the ability of the Ag to induce productive humoral and/or cellular adaptive immune response(s) in the host. It is determined by features of both the Ag and the inoculated host (Mahanty et al., 2015). Obviously, immunogenicity is a necessity of vaccines, while it is unwanted in therapeutic proteins. Therefore, the immunogens that produce optimal protective immune responses are selected for development of vaccines.

Development of modern rational vaccines is a multiphasic process that involves identification of candidate vaccine immunogens in the preclinical phase. Structural biology of B and T cell epitopes, 'omics' sciences, 'reverse' vaccinology and systems vaccinology approaches are increasingly being employed for selecting and designing 'rational' and 'individualized/tailored' vaccines with predictable outcomes (Adu-Bobie et al., 2003; Adams et al., 2011; Buonaguro et al., 2011; Furman & Davis, 2015; Lanzavecchiam et al., 2016; Singh, 2016; Charleston & Graham, 2018). 'Reverse vaccinology', starting from whole genome sequence of the pathogen, employs first *in silico* methods to predict potential B and T epitopes of immunogens of candidate vaccines, and then *in vivo, ex vivo* or *in vitro* immunogenicity tests (Adu-Bobie et al., 2003; De Groot et al., 2008; Cohen et al., 2010; Castelli et al., 2013; Sanchez-Trincado et al.,

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2017). Systems vaccinology employs systems level information on host-pathogen interactions (called interactomes), immunomics and computational biology tools to design rational (sometimes called tailored or personalized) vaccines with predictable immune outcomes in individuals (Adams et al., 2011; Buonaguro et al., 2011; Pulendran, 2014; Swann et al., 2015).

Immunogenicity of any type of vaccine is best demonstrated by protection of the animal model or the target host against pathogen challenge (Kumar and Singh, 2005; Herath et al., 2010), but animal ethics and costs remain a concern. Moreover, inclining trends of animal welfare concerns have given impetus to replace, reduce and refine the use of animals at all stages of vaccine development and evaluation (Singh, 2018). As an alternative to pathogen challenge studies in the host, *ex vivo* measurement of correlates of protection or *in vitro* surrogates of B and T cell immunity (Plotkin, 2010; Thakur et al., 2012; WHO, 2013), if already validated for the vaccine, save time and costs, and improve animal welfare.

Various phenotypic and functional parameters of the humoral and cellular immune responses can represent the potential correlates, co-correlates or surrogates of protection (De Groot et al., 2008; Plebanski et al., 2010; Amanna & Slifka, 2011; Saade et al., 2012; WHO, 2013). These immune parameters can be determined outside animals, i.e., *ex vivo* or *in vitro*. Both primary and recall immune responses can be examined by employing samples of whole blood, peripheral blood mononuclear cells (PBMCs), or isolated lymphocyte subsets from vaccinated and control animals, or *in vitro* Ag-primed monocyte-derived dendritic cells (MoDCs)-co-cultured with PBMCs or isolated T cells.

Immunogenicity testing needs to be focused on measuring valid correlates of vaccine-induced protection or surrogates of B and T cell immunity in order to improve efficacy evaluation of commercial vaccines as well as identification and selection of immunogens in the preclinical phase of rational vaccine development. Unfortunately, valid correlates of protection or surrogates have been established only for a limited number of veterinary vaccines, because of limited availability of species-specific reagents, somewhat relaxed regulatory requirements, and slow pace of expansion of the species-wise immunology knowledge-base (Knight-Jones et al., 2014; Veterinary Vaccinology Network meeting, 2017; Kapoor et al., 2017). The aims of this article are to review the progress in development of *ex vivo* and *in vitro* tests that measure various immune parameters as potential correlates or surrogates of vaccine-induced protection, and to promote the development of a network for research in this field of veterinary vaccinology.

Determinants of immunogenicity

As stated above, characteristics of both the Ag and the host determine the outcome of immune responses, i.e., productive (Ab, effector T cells, memory B and T cells) or null (Ag-specific B or T cell tolerance). Ag characteristics include dose, chemical nature and composition, physical form, associated danger/adjuvant, processibility, etc. Host characteristics include age, pre-inoculation immune status, route and anatomical site of Ag inoculation, major histocompatibility complex (MHC) haplotype and other genetic background, physiological and nutritional status.

Proteins are the best characterized of all chemical types of Ags and often chosen as immunogens for developing rational vaccines. They possess two types of small-sized epitopes, also called Ag determinants: B epitopes bound specifically by Ag receptors on B cell clones and T epitopes bound as MHC-Ag peptide complex by Ag receptor on T cells. An Ag can possess multiple B and T epitopes, and the immunodominant ones need to be identified and incorporated in candidate vaccines. Bioinformatics tools for accurate prediction of B and T epitopes are now available, and are being refined for accuracy of prediction (Cohen et al., 2010; Castelli et al., 2013; Lanzavecchia et al., 2016; Sanchez-Trincado et al., 2017). However, the predicted epitopes need to be experimentally validated for their immunogenicity.

The need and rationale of vaccine immunogenicity testing outside animals

Immunogenicity of both candidate and commercial vaccines can be tested in or outside animals. In animals, protection against pathogen challenge is observed by prevention of disease,

infection or clinical severity as readout. Though the best and traditionally followed for both preclinical vaccine development and efficacy evaluation of most commercial vaccines as a matter of regulatory requirement, it is the costliest and ethically questionable method. Alternatively, immunogenicity can be tested by measuring immune correlates or surrogates of protection outside animals. This method saves time and costs, and improves animal welfare, but requires that the immune correlates of vaccine-induced protection or surrogates of B and T cell immunity have been previously determined.

Availability of valid correlates or surrogates of protection as substitute end points are important for vaccine development, licensure and effectiveness monitoring. It is possible to establish correlates or surrogates of vaccine-induced protection, because of interrelationship of vaccination, the immune response, protection, and clinical outcomes (WHO, 2013). However, the terms such as correlates, surrogates, co-correlates, protection, etc. need to be defined and used uniformly by all, including regulatory authorities for the purpose of research, development, licensure and monitoring of vaccines (Plotkin, 2010; WHO, 2013; Knight-Jones et al., 2014). Moreover, discovery of immune markers and valid correlates of protection for each human and veterinary vaccine will maximize the utility of the *ex vivo/in vitro* immunogenicity tests.Plotkin's general principles of vaccine-induced protection (as outlinedin Table 1) should guide the development and validation of *ex vivo/in vitro* tests for primary and recall immune responses that give the best or optimum estimates of vaccine immunogenicity.

Table 1: Plotkin's general principles of vaccine-induced protection

S. No.	Principle
1	The mechanism of protection is not necessarily the mechanism to control the infection, e.g., Abs prevent infection and cellular immune responses control virus infection.
2	Large challenge dose may overwhelm vaccine-induced protection.
3	Immune system has evolved to be redundant: existing licensed vaccines protect through antibodies, but multiple mechanisms of protection might work together.
4	Memory induced by vaccine correlates with protection, particularly for long incubation diseases.
5	Correlates may vary in individuals according to age, gender, MHC haplotypes, etc.
6	Correlates may vary qualitatively and quantitatively, depending on the goals to be achieved by vaccination, i.e., prevention of systemic or mucosal infection, disease or clinically severe disease.

Correlates of protection or *in vitro* surrogates have been validated for some commercial human vaccines and are being investigated for others (Thakur et al., 2012). Humoral immune parameters are better understood and standardized than the cellular immune parameters as correlates or surrogates of protection by commercial vaccines. Neutralizing Abs, serum IgG or mucosal IgG and IgA are potential correlates of protection induced by some human vaccines. Both Ab titres and Ab classes or subclasses may be important. For example, 1000 IU/ml and 0.1 IU/ml of toxin neutralizing Abs in sera from vaccinated individuals correlate with protection induced by anthrax and tetanus vaccine, respectively (Thakur et al., 2012). Similarly, protective levels of neutralizing Ab titres in sera of individuals vaccinated with smallpox, Japanese encephalitis, and yellow fever vaccines are known. Protective Ab titres determined by ELISA are established for hepatitis A, hepatitis B, Hib conjugate, Hib polysaccharide and other vaccines. Like-wise, it is feasible to establish correlates of protection for veterinary vaccines as well.

Cell-mediated immunity is necessary for protection against chronic diseases, intracellular

pathogens and cancers. Therefore, T cell parameters are likely to be significant correlates of protection, dictating the identification and selection of immunogens for vaccines against these diseases. Several markers or parameters of cellular immune responses that have been investigated as potential correlates of protection are: i) IFN-³, IL-2, TNF± (Th1 signatures), ii) IL-1, IL-4, IL-10 (Th2 signatures), iii) IL-17 (Th17 signature), iv) $CD27^{+}FCRL4^{+}$ memory B cells, v) $CCR27^{hi}CD45RA^{+}$ $CD27^{+}CD28^{+}T_{CM}$, vi). $CCR27^{lo}CD45RA^{-}CD27^{+/-}CD28^{+}T_{EM}$, vii) $CD3^{lo}CD56^{+}$ NKT cells, etc. For example, IFN-³ production by BCG vaccine and $CD4^{+}$ cells and lymphoproliferation by zoster vaccine have been found to correlate with protection in humans (Plotkin, 2010).

With further advancements in understanding of species-level features and functions of immune systems and host-pathogen interactomes, immune markers and valid correlates of protection should become available for screening of immunogens by the *ex vivo/in vitro* tests.

The *ex vivo* and *in vitro* tests that measure humoral and cellular immunity parameters as potential correlates or surrogates of vaccine-induced protection

As described above, many different *ex vivo/in vitro* immune parameters of humoral and cellular immunity that can be potential correlates, co-correlates or surrogates of vaccine-induced protection are briefly introduced below. Detailed methodology of these tests is available in the scientific literature (Crowther, 2001; Kalyuzhny, 2005; Plebanski et al., 2010; Saade et al., 2012; Vandebriel & Hoefnagel, 2012; Smith et al., 2015; Singh, 2017; Coligan et al., 2018).

A. Antibody measurement assays

ELISA, virus/toxin neutralization, hemagglutination inhibition, and other assays have been employed to measure levels of Abs of different classes and subclasses as correlates of protection (Crowther, 2001; Thakur et al., 2012; Singh, 2017). Levels of serum or mucosal Abs induced by most of the currently used vaccines are validated as correlates or co-correlates of protection against disease, infection or clinical severity. Other sensitive immunoassays for Ab quantification might also be developed. Next generation immunoassays, such as immunosensors and bead-based multiplex assays, etc. might also find place in this list (Singh, 2017).

B. B cell assays

ELISPOT assay is used to enumerate B cells as Ag-specific Ab-producer clones (Czerkinsky et al., 1983; Crotty et al., 2004). Immunophenotyping of memory B cells involves fluorescent Ab staining of memory B cell surface markers followed by flow cytometry (Coligan et al., 2018). Next generation assays such as microfluidics-based lab-on-a-chip (LOC) and AIS could be suitable for such purposes in future (Warren et al., 2010; Singh, 2017).

C. T cell assays

Different types of T cells, such as Th, Tc, memory T, and Tregs, and their subsets having different immune functions have been identified (Moser & Leo, 2010; Abbas et al., 2018), and their roles in protection investigated. Several tests that measure various T cell immunity parameters as potential correlates of protection are available (Plebanski et al., 2010; Saade et al., 2012; Vandebriel & Hoefnagel, 2012), and still others are being developed. Various categories of such tests are listed below:

- **T cell proliferation assays**

Several categories of lymphoproliferation assays exist. ^{3}H-thymidine uptake is the gold standard for measuring proliferative response (Brunner et al., 1968; Saade et al., 2012; Coligan et al., 2018). Alternative non-radioisotope tests have been developed, such as CFSE fluorescent dye dilution-based cytometric assays (Lyons, 2000), detection of BrdU incorporation in nascent DNA by using anti-BrdU mAb staining and flow cytometry (Leif et al., 2004), and Ki67 nuclear Ag marker of dividing cells for detection *ex vivo* by anti-Ki67 mAb staining and flow cytometry (Shedlock et al., 2010).

- **Cytokine measurement assays**

ELISA for quantifying cytokines as signatures of T cell subsets [IFN-³, IL-2, TNF± (Th1

signatures); IL-1, IL-4, IL-10 (Th2 signatures); IL-17 (Th17 signature)] are the most widely used conventional gold standard and available as kits (Crowther, 2001). But, more sensitive multiplex cytometric bead assay, a flow cytometry-based assay, is now able to measure multiple cytokines and chemokines e.g., Luminex xMAP® technology (Defawe et al., 2012).

- **Ag-specific T cell enumeration**

ELISPOT assays are often used for enumeration of lymphocyte populations that secrete cytokines, antibodies, granzymes or perforins (Kalyuzhny et al., 2005; Goodell et al., 2007; Lehmann & Zhang, 2012). Several other tests have been developed, namely, fluorospot assay for detection of multiple cytokines per cell (Ahlborg & Axelsson, 2012), intracellular cytokine staining (ICS) and flow cytometry (Lovelace & Maecker, 2011; Smith et al., 2015; Yin et al., 2015), cytokine secretion assay for live T cell counting by flow cytometry (Brosterhus et al., 1999), *in vitro* tetramer staining of cytotoxic T lymphocytes (CTLs)and Th cells for flow cytometry (Gallimore et al., 1998; Novak et al., 1999).

- **Cytotoxic T cell assays**

Traditional ^{51}Cr release assay has been used since long to measure CTL activity *in vitro* (Brunner et al., 1968; Bachy et al., 1999). Several non-radioactive CTL cytotoxicity assays have been developed, namely, assaysto detect the markers of degranulation of CLTs, such as lactate dehydrogenase or ^{2}-galactosidase (Decker & Lohmann-Matthes, 1988; Bachy et al., 1999), europium labeled or bicistronic vector transfected target cells (Cui & Bystryn, 1992; Gupta et al., 2009), indicators of apoptosis/necrosis of target cells (Memon et al., 1995; Wlodkowic et al., 2011), effector cell degranulation by anti-CD107a (Alter et al., 2004; Wlodkowic et al., 2011), or CTL granzymes/ perforin- ELISPOTs (Zuber et al., 2005).

- **T cell immunophenotyping**

Central memory T cells (T_{CM}), restricted to lymphoid tissues, and effector memory T cells (T_{EM}), circulating in non-lymphoid tissues, correlate with protection respectively by vaccines against long-incubation diseases and short-incubation diseases (Reece et al., 2004; Pepper & Jenkins, 2011). These subsets have been detected by immunophenotyping and flow cytometry (Altman et al., 1996; Waldrop et al., 1997; Maecker, 2009; McKinnon, 2018).

- **T cell signaling based assays**

Detection by intracellular staining of key molecules in signaling pathways such as phosphorylated ERK, ZAP70, NFkB or STAT5 in $CD4^+$ T cells is done to indicate early clues to the effectiveness of a vaccine (Downey et al., 2011; Puronen et al., 2012).

- **Multi-parametric T cell assays**

Cytometry by time-of-flight mass spectrometry (CyTOF-MS) is a new method that can measure up to 60 parameters at a single-cell level, including surface markers, intracellular molecules (cytokines & others), activation markers, etc. (Newell et al., 2012).

- ***In vitro* MoDC-lymphocyte co-cultures**

Currently, *in vitro* Ag-primed MoDC: T cell co-culture models are being standardized and validated for screening immunogens of candidate vaccines and also evaluating efficacy of vaccines. DCs can be differentiated *in vitro* from CD14+ monocytes, which are isolated from peripheral blood and cultured in the presence of GM-CSF and IL-4 (Clinton & Shapiro, 2015; Park et al., 2016; Sim et al., 2016; Lutz et al., 2017). MoDCs have $CD14^+CD11a\text{-}c^+CD1a^+CD16^+CD40^+CD80^+$ $CD86^+CD163^+CD172a^+CD205^+CD206^+CD209^+$ $MHC\text{-}II^+$ phenotype. MoDCs have been used to demonstrate *in vitro* processing and presentation of Ags of two candidate vaccines of *Mycobacterium avium paratuberculosis* and to elicit a recall response in $CD4^+$ and $CD8^+$ T cells from a vaccinated steer (Park et al., 2016).

D. Artificial immune system

Immune system *in vitro,* where DCs as APCs, B and T lymphocytes can interact and generate immune responses in a 2D co-culture system has been constructed. AIS can be used for immunogenicity testing *in vitro* for vaccines,

therapeutic proteins and other biologicals (Warren et al., 2010).

Concluding remarks

Ex vivo and *in vitro* immunogenicity testing is aimed at saving costs and time for vaccine development and evaluation, and improving animal welfare. With technologies in reach, it is time to create networks for initiating discovery and validation of immune markers as correlates of protection induced by current and novel veterinary vaccines against major diseases of domestic animals, including zoonosis and food safety. The availability of these immunoassays will impact the way the veterinary vaccines will be discovered, designed and evaluated in the future.

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Determination of Immune Related Haemato-biochemical Parameters of Native Chicken of Himachal Pradesh and Comparison with Dahlem Red Breed

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ABSTRACT

Native chickens are the important part of the free range and scavenging poultry production of rural areas of the Himachal Pradesh. The knowledge of baseline values of immune related haemato-biochemical parameters under local conditions is essential. Thus, haematological parameters such as heterophil, lymphocyte, basophil, eosinophil and monocyte counts and biochemical parameters such as plasma total proteins, albumin and globulin for native chicken of Himachal Pradesh were estimated and compared with the exotic breed Dahlem Red. In native chicken; heterophil, lymphocyte, basophil, eosinophil and monocyte counts were not found to be significantly ($p>0.05$) different from the exotic breed, Dahlem Red. Plasma total proteins and albumin were found to be significantly ($p<0.05$) different but, no significant difference ($p>0.05$) for globulin was observed between the native and exotic breed, respectively.

Keywords: Native chicken, Dahlem Red, haemato-biochemical parameters

INTRODUCTION

Poultry farming has made an important place in the livestock industry of India by raising gross domestic product (GDP) and completing the protein requirements of the people through meat and eggs (Ullengala *et al.*, 2008). Free range poultry production as well as scavenging poultry production is dominated by native chicken in rural areas of Himachal Pradesh. Native chicken of Himachal Pradesh comprise of non-descript breed, usually brown in colour with adult male and female body weights as 1.2(±0.03) and 1.8(±0.05) kg, respectively exhibiting late maturity and low production (Sankhyan *et al.*, 2013). These birds show good foraging and efficient mothering character with adaptation to the local environment and effective resistance against the diseases (Reen *et al.*, 2014) but, are not as effective producers as the commercial lines. Dahlem Red, an exotic improved layer breed is used for cross-breeding of native chickens in Himachal Pradesh for improved characters in the filial generations which combine the quality of both native and exotic breed used (Sankhyan *et al.*, 2013).

Serum/plasma biochemical and haematological parameters provide insight to the immune status and this type of information is important for disease diagnosis and optimal management. Differential leucocyte count is the common indicator of haematological, immunological and infection status of the individual (Harr *et al.*, 2009). Leucocytes, particularly neutrophils and macrophages primarily defend the body by phagocytosis of invading infectious agents and form the innate part of immune system (Coles, 1986; Tseke, 2010). Blood biochemical estimation along with these parameters helps to know about pathological process and proper functioning of various vital organs (Harr, 2009 and Ceron *et al.*, 2010). This information can equally be used by the breeders during the genetic improvement of the native chickens under various breeding programs (Kral and Suchy, 2000). Currently there is no information available about immune related haematological and biochemical parameters of native chicken of Himachal Pradesh. Thus the objective of this study was to establish baseline data for these native birds along with a comparison with the exotic chicken breed, Dahlem Red.

MATERIALS AND METHODS

Location of study

The study was conducted at poultry farm of College of Veterinary and Animal Sciences, CSK HPKV, Palampur. It is located at 32.1º N

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latitude, 76.5º E longitude and 1290.8 m altitude. The average annual rainfall is between 2100-2200 mm. The average temperature is around 26º C during summer season and 14º C during winter season.

Birds and management

Two breed-wise groups, each comprising of twenty adult birds with equal numbers of males and females of native and Dahlem Red breeds were constituted. Birds were randomly selected and maintained on deep litter with nest boxes. Birds were fed *ad libitum* crumbled feed according to their age and feeding requirement. Leg bands were used for identification of the selected birds.

Blood collection

Blood samples were collected at 25 weeks of age from all the experimental birds with sterile 24-gauge needles by aseptic venipuncture of ulnar vein. In total, 0.5 ml of blood was collected in the ethylene diamine tetra acetic acid (EDTA) coated vials for haematological analysis and 2 ml of blood was collected in heparinized vials for biochemical analysis.

Differential leucocyte count (DLC) estimation
Fresh blood smear was prepared and stained with the Wright's stain (Qualigens Fine Chemicals, GlaxoSmithKline Pharmaceutics Ltd). Counting was done with the help of blood cell counter which had a separate key for each type of blood cell.

Blood biochemical parameters estimation

Total proteins and plasma albumin proteins were estimated by using biochemical estimation kit (Agappe diagnostics Ltd.) in an automatic blood biochemistry analyzer (Mispa Nano, Agappe). Globulin content of the plasma samples was estimated by subtracting albumin content of the sample from the corresponding total protein content.

Globulin content (g%) = Total protein (g%) - albumin (g%)

Statistical analysis

The haemato-biochemical values obtained were statistically analysed by GraphPad InStat software (GraphPad InStat, 2008). The data was subjected to one-way analysis of variance (ANOVA) using Tukey's test at five percent level of significance.

RESULTS AND DISCUSSION

Review of the literature shows no studies on the immune related haematological and biochemical parameters of native chicken of Himachal Pradesh. The haematological and biochemical parameters for native and exotic chicken are shown in the Table 1. No significant difference was observed among the haematological parameters of the native chickens, but significant difference ($p<0.05$) was found for the biochemical parameters such as total protein and albumin as compared to Dahlem Red breed. Most predominant leucocyte found was lymphocyte in both the native and exotic birds which is similar to the findings of Prahsanth *et al.,* (2012) and Pandian *et al.,* (2012). Lymphocytes play important role in humoral and cell mediated immune responses (Ono *et al.*, 2003; Latimer and Bienzle, 2010). Heterophils are the phagocytes that regulate the innate host defense within the chickens by preventing tissue trauma, inflammation and destroying invasive microorganisms by phagocytosis and result in effective non oxidative bacterial killing (Evans *et al.*, 1995; Kogut *et al.*, 2002; Latimer & Bienzle, 2010 and Wakenell, 2010). Heterophil count in native birds were similar to that observed by Bora *et al.,* (2017) but higher than that observed by Abdi-Hachesoo *et al.,* (2011) and Jaiswal *et al.,* (2017), respectively for broiler chickens and lower than that observed by Obese *et al.,* (2018) for guinea fowl. During early inflammation and hypersensitivity reactions, basophils in avians serve an important role (Maxwell and Robertson, 1995). The basophils in native chicken and Dahlem Red were found to be similar to those observed by

Table 1: Haemato-biochemical parameters of native chicken and Dahlem Red

Parameters	Native chicken	Dahlem Red
Lymphocytes (%)	51.00±0.68	41.00±0.80
Heterophils (%)	40.80±0.67	49.90±1.19
Basophils (%)	1.60±0.16	2.30±0.33
Eosinophils (%)	3.20±0.24	3.50±0.37
Monocytes (%)	3.40±0.22	3.30±0.26
Total proteins (g/dl)	4.39±0.21[a]	3.86±0.09[b]
Albumin (g/dl)	2.25±0.06[a]	2.08±0.03[b]
Globulin (g/dl)	2.14±0.20	1.79±0.08

Figures with different superscripts (a, b) differ significantly ($p<0.05$) between the two given breeds

Bora *et al.,* (2017), lower than basophils for the Thai indigenous birds and guinea fowl (Simaraks *et al.*, 2004 and Obese *et al.*, 2018) and higher than pheasant (Kececi and Col, 2009). Monocytes form the part of phagocytic system that includes connective tissue macrophages, osteoclasts, perisinusoidal macrophages and macrophages of lymph and bone marrow (Campbell, 1994; Teske, 2010). Eosinophils are the defenders of host against the helminth parasites and allergic reactions (Latimer and Bienzle, 2010). Eosinophil and monocyte counts in this study were similar to those found by Abdi-Hachesoo *et al.*, (2011). However, Pandian *et al.*, (2012) observed higher monocyte count for Rhode Island Red chickens but similar eosinophil count as seen for Dahlem Red chickens in this study. Obese *et al.*, (2018) observed lower eosinophil and monocyte count for the guinea fowl as compared to the birds in this study.

Native birds have significantly higher plasma total proteins and albumin content than the exotic chicken. The values observed were within the range observed by Simaraks (2004) and Pampori and Iqbal (2007) for native chicken of Kashmir. Plasma total proteins of Dahlem Red were slightly lower than those observed by Yadav (2018) and lower than indigenous birds observed by Ladokun *et al.*, (2008), Elagib *et al.*, (2012), Albokhadim *et al.*, (2012) and Srinivas and Swathi (2017). Albumin is the most predominant protein of plasma. Hepatocytes synthesize albumin and excrete it out into the blood circulation at the rate of 10 to 15 gm per day. Albumin maintains the plasma osmotic pressure and homeostasis, transport ligands along with providing insight of liver's ability to synthesize proteins. Native birds have significantly ($p<0.05$) higher albumin content than the Dahlem Red birds. The plasma albumin concentration observed in this study was higher than those observed for laying desi hens by (Bhatti *et al.*, 2002), Hybro PG broilers (Silva *et al.*, 2007) and semi wild indigenous chicken of Mizoram (Mayengbam *et al.*, 2017). However, the albumin concentration was found lower than pheasants (Kececi and Col, 2009), indigenous and broiler chicken of Iran (Abdi-Hachesoo *et al.*, 2011) and Aseel chicken (Rehman *et al.*, 2017). Plasma globulin consists of other serum proteins, enzymes and immunoglobulins that are synthesized in liver by the plasma cells. Usually the increase in immunoglobulins lead to increase in globulin content and immune deficiency and malnutrition can cause decreased globulin synthesis (Walker *et al.*, 1990). The plasma globulin content of native chicken in this

study was not significantly ($p>0.05$) higher than the Dahlem Red breed. The estimated values in this study were lower than those observed by Pampori and Iqbal (2007) and Mayengbam *et al.,* (2017) but higher than those observed by Albokhadaim *et al.,* (2012) and Ladokun *et al.,* (2008).These differences could be due to different genotype, feeding, environment and management conditions.

Conclusion

Baseline data is important to know about the health status of the individual. These immune related haematological and biochemical parameters will have direct application in the cross breeding programmes and evaluating the health and disease status of these chicken breeds.

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Conflict of interest

The authors do not have any conflict of interest.

Ethical approval

All procedures were followed for the ethical treatment of animals.

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Comparative Efficacy of Gamma Interferon Assay with Comparative Intradermal Tuberculin Skin Test in Diagnosing Tuberculosis in Dairy Animals

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ABSTRACT

Comparative merits of Comparative intradermal test (CITT) and Gamma interferon assay (IFN-^{3}) were assessed in detecting bovine tuberculosis (bTB) in dairy cows and buffaloes (n=201) having clinical symptoms and history suggestive of TB. Out of the 201 animals tested, 19.4%, 10% and 14.4% were positive for TB by CITT only, IFN-^{3} assay only and by both the tests, respectively. This clearly demonstrated that these tests, if performed individually, would not be enough to accurately detect TB in dairy animals. Instead, these would complement each other for efficient diagnosis of TB. Amplification of IS900 gene sequence specific for *Mycobacterium avium* subspecies paratuberculosis revealed 229 bp band in DNA extracted from the fecal samples of 120 animals. The percentage of Johne's disease was 12.5%.

Keywords: Gamma Interferon, Tuberculosis, Intradermal Tuberculin, Diagnosis

INTRODUCTION

Tuberculosis (TB) is an infectious, zoonotic disease of tremendous economic importance in dairy animals caused mostly by *Mycobacterium bovis*, and to some extent by *Mycobacterium tuberculosis.* Out of 54 Mycobacterium tuberculosis complex (MTC) isolates obtained during a study in dairy cattle in Northern India, 40 (74.07%) were of *M. bovis* and 14 (29.92%) were of *M. tuberculosis* (Srivastava et al. 2008). Bovine TB can also be transmitted to humans through close contact with animals, inhalation of infected droplets or aerosols, and infected animal urine besides drinking contaminated milk. There has been a direct correlation between *M. bovis* infection in cattle and the occurrence of TB in humans (Cosivi et al. 1998) probably due to drinking or handling of TB contaminated milk. High frequency of transmission of TB pathogens from humans to cattle and *vice versa* is suspected (Prasad et al. 2005). Bovine TB leads to severe economic losses due to variable decrease in milk yield (Hernandez and Baca 1998, Boland *et al.,* 2010). Various diagnostic methods for TB in bovines have been used, albeit with variable efficiency and reliability (Strain et al. 2011). Most of the hematological and biochemical studies conducted to evaluate their usefulness as diagnostic markers for TB remained inconclusive (Javed et al. 2006, 2010). Although the Single Intradermal Test (SID) and the Comparative Intradermal Tuberculin Test (CITT) are the most commonly used diagnostic tests for bovine tuberculosis (OIE 1992 and Radostits et al. 1994) however, these when conducted alone lack sensitivity and specificity (Wood et al.1991 and Fifis et al. 1994). The Gamma interferon (IFN-^{3}) assay has improved the diagnosis of TB by measuring the cell mediated immune response against *M. bovis* antigen (Wood 1989, Liebana et al. 1998 and Gormley et al. 2006). Further studies however, are required to properly understand the comparative merits of these tests for diagnosis of TB, especially in cows and buffaloes of various age groups, and those with different production and nutritional status. The present study was, therefore, undertaken to evaluate the comparative efficacy of IFN-^{3} with CITT in diagnosing TB in dairy cattle and buffaloes suspected for tuberculosis.

There are reports of high incidence of Johne 's disease (JD) in dairy animals in India (Singh et al. 2007, Chachra et al. 2012). Since concurrent infections of both TB and JD may affect the diagnosis of TB using IFN-^{3} (Wood et al. 1991, Liebana et al. 1998 and Alvarez et al. 2008), the

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incidence of JD was also studied in some of the animals tested for TB and its implications on occurrence or diagnosis of TB, if any, was evaluated.

MATERIALS AND METHODS

The study was conducted on 201 dairy animals (128 were cattle and 73 were buffaloes) housed at three dairy farms in and around Ludhiana city of Punjab, India. The animals enrolled in the study were having one or a combination of the various clinical signs suggestive of tuberculosis at the time of examination, such as chronic cough, weight loss, fever, anemia, nasal discharge, weakness etc. All the 201 animals were screened for tuberculosis using CITT and IFN-^{3} assay. Out of these, faecal samples of 120 animals were also tested for paratuberculosis. The animals were divided into four groups based upon their responses to CITT and IFN-^{3} assays alone and in combination as shown in Table 1

The Gamma interferon assay

IFN-^{3} estimation was done through an ELISA using *Mycobacterium bovis* Gamma Interferon Test Kit for Cattle (BOVIGAM) procured from Prionics, Switzerland. Briefly, blood samples 5ml were collected in heparinized vials from each animal through jugular vein-puncture, transported to the laboratory at ambient temperature (22°C) within 6 hours and stimulated with bovine and avian TB stimulation antigen (100 µl of 0.3mg/ml each; Prionics) for 24 h. The mean OD of nil, avian and bovine PPD were recorded and the values of avian and nil PPD were subtracted separately from the value of bovine PPD. Animals were considered positive when OD of bovine PPD - OD of avian PPD and OD of bovine PPD – OD of nil was > 0.1. Animals were considered negative when the OD of bovine PPD - OD of avian PPD and OD of bovine PPD – OD of nil was < 0.1

Intra-dermal tuberculin testing

Comparative Intardermal Tuberculin Test (CITT) was undertaken as per OIE Terrestrial Manual (1992). For CITT, bovine tuberculin purified protein derivative (PPD) from culture of *Mycobacterium bovis* (strain AN5, 3000 IU) and avian tuberculin PPD from culture of *Mycobacterium avium* subspecies *avium* (strain D4ER, 2500 IU), obtained from Prionics (Switzerland) were used for CITT. The animals were considered positive for TB if the increase in skin thickness at the site of bovine PPD injection was more than 4 mm than the reaction shown at the site of the avian PPD injection. The reaction was recorded as negative, if no or d4 mm difference in the increase in skin fold thickness was observed.

Statistical analysis

Kappa test (Winscope software) was used to assess the degree of agreement between the results of CITT and IFN-^{3}. The data were significant at $p<0.05$ or $p<0.01$.

Molecular diagnosis of paratuberculosis

DNA extraction from fecal samples (n=120) was done as per the method of van Embden *et al* (1993). The primers were species specific and based on the insertion sequence IS*900* (IS900 150C 5'- CCG CTA ATT GAG AGA TGC GAT TGG - 3'/ IS900 921 5'- AAT CAA CTC CAG CAG CAG CGC GGC CTC G -3') designed to amplify 229 bp target sequence (Vary *et al.,* 1990). Known positive DNA was obtained from Dr. Stevenson, UK. Thermal cycling was performed in thermal cycler with initial denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 45 sec, annealing at 63°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis. Amplicons of 229 bp were considered positive for IS*900* PCR.

RESULTS

A total of 201 dairy animals (128 were cattle and 73 were buffaloes) suspected for tuberculosis were tested using CITT and IFN-^{3} assay. Out of these 120 animals were also subjected to diagnosis of paratuberculosis. The animals were divided into four groups based upon their responses to CITT and IFN-^{3} assays alone and in combination as shown in Table 1. Based on the results of the CITT and the IFN-^{3} assay, the animals were divided into four groups (Table 1).

The percentage of animals tested TB positive with either or both the tests, was 43.8 per cent. This percentage was 45.3% in cows (45.3%) and 41.1% in buffaloes. Further, the proportion of cows and buffaloes tested positive by either both the tests (Group 1) or by the CITT alone (Group 2) were also almost the same. However, the proportion of TB positive buffaloes responding to IFN-^{3} assay were apparently lower than their counterpart cows (Group 3) (Table 1).

Table 1: Groups of animals tested for TB with CITT and the INF-^{3}

Group No.	Criteria	Number of Cows (%)	Number of Buffaloes (%)	Total Animals (%)
Group 1	Animals tested TB positive by both the tests i.e. CITT and IFN-^{3} Assay	17 (13.3%)	12 (16.4%)	29 (14.4%)
Group 2	Animals tested TB positive by CITT only	24 (18.8%)	15 (20.5%)	39 (19.4)
Group 3	Animals tested TB positive by IFN-^{3} Assay only	17 (13.3%)	3 (4.1%)	20 (10.0%)
Group 4	Animals tested TB negative by both the tests i.e. CITT and IFN-^{3} Assay	70 (54.7%)	43 (58.9%)	113 (56.2%)
Total TB positive	Number of animals tested TB positive with one or both the tests	58 (45.3%)	30 (41.1%)	88 (43.8%)

Intradermal tuberculin test

The differences in skin thickness after CITT were significantly higher ($P<0.05$) in Groups 1 and 2 of both cows and buffaloes than their respective counterpart animals in Groups 3 and 4. The cows and buffaloes which otherwise tested TB positive with IFN-^{3} (Groups 3 of each species) had differences in skin thickness similar to those animals which tested negative for TB by either of the two tests (Groups 4 of each species) as shown in Table 2.

The Gamma interferon assay

Out of 201 animals screened, 49 animals tested positive for IFN-^{3} (34 cattle, and 15 buffaloes). Out of these only 20 animals (17 cattle and 3 buffaloes) showed an exclusive positive reaction to IFN-^{3} and a negative response to CITT. The rest 29 animals showed a positive response to both IFN-^{3} and CITT. Out of 68 animals which tested positive with CITT (33.8%), only 29 (42.6%) were tested positive with IFN-^{3}. The rest 39 (57.3%) tested negative.

Comparative evaluation of CITT and INF-^{3}

The compatibility or agreement between the efficiency of CITT and that of IFN-^{3} remains to be established. To compare the degree of agreement between the two tests, Kappa test was applied statistically. At 95% level of significance, kappa value between intradermal and gamma interferon test was calculated to be 0.296 which indicated a fair degree of agreement between the two tests.

Effect of JD on efficacy of CITT and INF-^{3} TB screening tests

Out of the 120 animals tested for JD by PCR, only fifteen animals (12.5 percent) revealed the 229 bp band amplifying the IS900 sequence of *Mycobacterium avium paratuberculosis* (Table 3). Only eight animals were positive for JD in the group of animals which were found positive for bTB by CITT alone, IFN alone or positive by both the tests, while seven animals were positive

Table 2: Difference in skin thickness (mm) after intradermal inoculation of avian and bovine TB antigens

Species	Group 1	Group 2	Group 3	Group 4
Cattle (n=128)	7.253 ±1.1216[a] (n=17)	7.492 ±0 .6544[a] (n=24)	0.894 ±0 .5432[b] (n=17)	1.449 ±0 .216[b] (n=70)
Buffaloes (n= 73)	7.408 ±0 .7008[a] (n=12)	7.14 ±0.756[a] (n=15)	0.467 ±1.8667[b] (n=3)	0.23 ±0.4811 [b] (n=43)

Means with different superscript (a and b) in a row differ significantly ($P<0.05$)

for JD in the group of animals which were negative for bTB in either of the tests.

Irrespective of the TB status of the tested animals, the incidence of JD ranged between 8-19 percent in the various groups as shown in Table 3 below. There was no apparent relationship between the occurrence of TB revealed by either of the tests and the proportion of animals positive for JD (Table 3).

Table 3: Incidence of JD in various groups of TB tested in dairy animals

Animals in various TB tested groups	No of animals tested positive for JD	Proportion of animals tested positive for JD (%)
Positive by CITT and Negative for IFN- ³ (n=16)	3/120= 2.5%	3/16=18.8%
Positive by IFN- ³ and Negative for CITT(n=16)	3/120= 2.5%	3/16= 18.8%
Positive for CITT and IFN- ³ (n=24)	2/120= 1.6%	2/24= 8.3%
Negative by CITT and IFN- ³ (n=64)	7/120= 5.8%	7/64=10.9%
Total	15/120= 12.5%	

DISCUSSION

High prevalence rates of bovine TB in an earlier study conducted in a small cohort of dairy animals in one of the states in India have recorded it to the tune of 14.31 to 34.42 percent (Thakur et al. 2010). Higher number of TB animals in the present study could be due to a biased selection of test population already suspected to have TB. SID

has a drawback of high rate of false positive results because of mycobacterial antigens that are not specific for *M. tuberculosis* / *M. bovis*. CITT is more specific than the SID test as it compares the animal's reactivity of bovine tuberculin to an avian tuberculin, thus distinguishes infection with *M. bovis* from that of *M. avium* or *M. paratuberculosis* (OIE 1992, Radostits et al. 1994, Gutierrez et al. 1998 and Schiller et al. 2010). In CITT, lack of significant differences in skin thickness in Group 3 cows suggests that the TB positive animals in Groups 3 might have been in early stages of the TB which could not be reflected in CITT. Neill et al. 1994 had also reported that gamma interferon assay could detect *M. bovis* cattle which were SID negative. Even weak immune system has also been held responsible for the low sensitivity and specificity of tuberculin testing in animals (Ozturk et al. 2010). The interferon gamma assay is an *in vitro* cellular assay to detect IFN- cytokine which is involved in immunity to mycobacterial infections

and it is used as a routine diagnostic test for TB. Gormley et al. 2006 stated that strategic use of the IFN-³ assay (Bovigam) could provide a means for the early identification of *Mycobacterium bovis* infected cattle, thus ensuring their removal from an infected herd. When used in parallel with the tuberculin test, it is capable of identifying infected cattle, which might otherwise not be detected until later, if at all. Results from experimental infections of cattle indicated that the assay could detect infection as early as 14 days post-infection and earlier than the tuberculin test (Buddle et al. 1995). The test sensitivities in infected herds have been reported to lie between 55 and 97 percent depending on the interpretation used, while specificities as high as 97 percent have been determined in tuberculosis-free herds (Monaghan et al. 1997). The fact that 20 animals which tested negative for TB with CITT were positive with IFN-³ suggested that these animals might have been in the early stages of the disease which couldn't be determined by CITT as also observed by other workers (Gormley et al. 2006, Coad et al. 2008, Strain et al. 2011). Gormley et al. (2004) reported that animals which tested positive for IFN-³ assay and negative for intradermal tuberculin test subsequently converted to tuberculin positive and posed an increased risk to the other cattle. IFN- ³ negative and CITT positive results may be caused by co-infection of the animal with an environmental mycobacterium or anergic situation of infected animals (Gormley et al., 2004 and De la Rua Domenech et al. 2006).

Both tests when used simultaneously increased the detection of maximum number of TB reactor dairy animals especially in endemic areas as also reported by Bassessar et al. 2014. Ameni et al. (2000) reported that there was no significant difference between the sensitivity and specificity of CITT and IFN-³, thus concluding that the choice between the two tests depended on their cost, simplicity, livestock management and time factors rather than on their respective diagnostic value. In contrast the sensitivity of the IFN-³ assay was shown to be significantly higher than the single intradermal tuberculin test (Wood et al. 1991, Liébana et al. 1998, Wood and Jones 2001). A maximum overall sensitivity however (95.2%), was claimed to have been obtained by testing with the IFN-³ and the tuberculin test in parallel (Wood et al. 1991, Liébana et al. 1998, Gormley et al. 2006). Previous records showed that the sensitivity of IFN-³ varied between 81.8 and 100 percent for culture-confirmed bovine TB and specificity between 94% and 100% (Wood and Jones 2001) whereas the sensitivity and specificity of single intradermal test was 68–95 percent and 96–99 per cent, respectively.

Occurrence of JD in the subject animals is not likely to have affected the accuracy of the tests used for testing TB. Some earlier studies have reported that sensitivity of IFN-³ decreased with the presence of corresponding paratuberculosis infection (Wood et al. 1991, Liebana et al. 1998 and Alvarez et al. 2008). Similarly Alvarez et al. 2009 found that for cattle co-infected with *M. avium paratuberculosis* the sensitivity of the IFN-³ test to detect *M. bovis* was 50 percent compared to 78 percent in *M. bovis* only infected cattle. This opinion however, was not corroborated by the findings of the present study as almost equal numbers of animals were found to be positive for JD in bTB reactors as well as in non reactors. Accordingly, the presence of JD in animals did not appear to have had any effect on the results of the other tests.

Conclusions

In conclusion, it was not possible to assign clear merits of detecting TB to either of the tests over the other. However, a combination of CITT and IFN-³ would complement each other in detecting TB in dairy animals especially in endemic areas. Further, the occurrence of JD in some of the TB positive animals did not seem to have affected the efficiency of the TB screening tests under study rather indicated the co-infections.

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Conflict of Interest

The authors declare they have no conflict of interest.

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Genotypic and Phenotypic Correlation of Antimicrobial Resistance in Bacterial Isolates from Mastitic Milk

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ABSTRACT

In the present study, milk samples from mastitic animals were collected and screened for the isolation and identification of bacterial etiological agents. Out of a total of 102 milk samples, 50 samples yielded bacterial growth. The bacteria identified were *Staphylococcus* (43), *Streptococcus* (15), *E. coli* (26) and *Klebsiella* (19). All the bacterial isolates were subjected to antibiotic sensitivity testing. Further, DNA from these bacterial isolates was isolated and subjected to PCR using genus specific primers which revealed that all the isolates were conforming to their respective genus. Also, DNA was subjected to PCR to detect the presence of *aac (3)-iv*, *bla*SHV, *aad*A and *tet*B, *tet*M, *erm* A, *mec* A and *bla*Z, *tet*M, *erm*B, *bla*Z and *mef*A antibiotic resistance genes revealed that there was an absolute correlation between the genotypic and phenotypic results of the antibiotic resistance.

Keywords: Mastitis, milk, PCR, antibiotic resistance genes.

INTRODUCTION

Mastitis is a very important disease which causes huge economic loss to the farmers due to loss of production. Various etiological agents (*Staphylococcus*, *Streptococcus*, *E. coli*, *Klebsiella* etc) have been implicated in causing mastitis. The most common practice to treat mastitis is by the use of antibiotics which has led to the development of antimicrobial resistance (Hogeveen et al. 2011).

The development of antimicrobial resistance is mediated by several mechanisms such as enzymatic inactivation or modification of antimicrobials, impermeability of the bacterial cell wall or membrane, active expulsion of the drug by cell efflux pump, alteration of target receptors, and drug trapping or titration (Guardabassi and Courvalin 2006).

The ability of the bacteria to share the genetic information proficiently enables them to survive in the presence of antimicrobials (McDermott et al. 2002) and the two most important ways by which bacteria acquire resistance are mutation or horizontal gene transfer (Van den Bogaard and Stobberingh 2000). These mechanisms increase the possibility of spread of antimicrobial resistance determinants from commensal organisms to the pathogenic (Salyers and Cuevas 1997).

Since mutation being a spontaneous process leads to structural and/or molecular changes and as a result, there is resistance development where mutants persist in a population with or without selection pressure from antimicrobials (Guardabassi and Courvalin 2006; Boerlin and White 2013).

In horizontal gene transfer mechanism resistance genes from donor to recipient are transferred utilizing either of transformation, transduction or conjugation (Schwarz and Chaslus-Dancla 2001). The mechanism of resistance or the resistance genes can be same for a variety of bacteria or limited to certain bacterial species or genera (Schwarz et al. 2006). Since, antibiotic resistance is a dynamic phenomenon in mastitis causing organisms (Singh and Baxi 1982; Makovec and Ruegg 2003), quick identification of the causative agent and then knowledge of antibiotic resistance genes could be of great help in prompt and successful treatment. Keeping this in view, the present study was envisaged with an objective to identify antibiotic resistance genes in mastitis causing organisms.

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MATERIALS AND METHODS

Collection of samples

Milk samples (102) suspected of mastitis (subclinical and clinical) were collected aseptically (after discarding first few streaks of milk) from the dairy farms in Ludhiana, Punjab after taking necessary permission from the Institution Animal Ethics Committee (IAEC). These samples were kept immediately on ice and transferred to the laboratory and subjected to bacterial isolation, identification, culture sensitivity test and extraction of DNA.

Isolation of bacteria

Milk samples were inoculated at 37ºC for 16-24 hours on Brain Heart Infusion (BHI) Agar, Eosin Methylene Blue (EMB) Agar, MacConkey's Lactose (MLA) Agar, Baird Parker Agar (BPA) supplemented with egg-yolk tellurite emulsion, Edward's medium and Blood Agar (BA) individually. Bacterial colonies after incubation were subjected to Gram's staining for identification and subjected to various biochemical tests for confirmation. After the biochemical confirmation the isolates of *Streptococcus, S. aureus, Klebsiella* and *E. coli* were sub cultured on BHI agar slants and preserved at 4^{o}C for further use. Also, a loop full of the isolated organism was added to the sterile nutrient glycerol (30%) broth vials, mixed well, labelled and preserved at -20ºC.

Antibiotic sensitivity test

All the isolates were tested for sensitivity to various antibiotics as per the disc diffusion methods of Bauer et al. (1966). Different antibiotics used were erythromycin (15mcg), methicillin (10mcg), penicillin (1U), streptomycin (10mcg), tetracycline (30mcg), and gentamicin (10mcg) (Himedia, Mumbai). In brief, overnight grown culture of individual bacteria in LB broth was uniformly spread onto Muller Hinton Agar plate with the help of a sterilized cotton swab. Antibiotic discs were placed at an appropriate distance and incubated for 16-24 hours. The zone of sensitivity and resistance was considered as per the CLSI, 2015.

Extraction of DNA

The DNA was extracted from the bacteria using Phenol: Chloroform: Isoamyl alcohol (PCI) as per the method of Sambrook and Russell (2001). In brief, a single isolated colony of the identified bacterium was grown overnight in LB broth. Next day, 1.8 ml of overnight incubated LB broth was taken in a 2 ml micro centrifuge tube (MCT) and centrifuged at 10,000 x *g* for 10 minutes. After discarding the supernatant, 50µl each of 10% SDS (Sodium Dodecyl Sulphate) and Proteinase K (200µg/ml) were added to the pellet and incubated in a water bath at 60°C for an hour. Later, 500µl of PCI (25:24:1) was added to the lysate and mixed by vortexing followed by centrifugation at 10,000 x *g* for 10 minutes. The supernatant was collected and the above step was repeated. To the supernatant, isopropanol and 3M sodium acetate (pH 5.2) (50:50) were added to the one-tenth volume of supernatant, mixed and kept at -20°C for overnight incubation. Next day, the pellet was collected after centrifuging it at 10,000 x *g* for 20 minutes. The pellet was washed twice with 500µl of 70% ethanol and reconstituted in 50µl of nuclease free water (NFW) and stored at -20^{o}C for further use. The purity and concentration of the extracted genomic DNA were estimated by using a Nanodrop (Thermo Scientific, USA). The optical density (OD) at 260 nm and 280 nm of the individual sample was measured by using nuclease free water as blank and the ratio of 260/280 OD was calculated. A ratio of 1.7 to 1.9 was considered satisfactory.

PCR

Polymerase chain Reaction (PCR) was carried out using genus specific primers and were synthesized from (Flarebio Biotech Inc., China for *E. coli, Klebsiella, Staphylococcus* and *Streptococcus* (Table 1). Further, all the isolates were subjected to PCR to detect the presence of *aac (3)-iv*, *bla*SHV, *aad*A and *tet*B, *tet*M, *erm* A, *mec* A and *bla*Z, *tet*M, *erm*B, *bla*Z and *mef*A antibiotic resistance genes (Table 2).

The PCR reaction for various antibiotic resistance genes was optimized individually. A PCR reaction mixture constituting 1X PCR buffer, 1.5mM $MgCl_2$, 200 µM of each dNTP,

one unit of Taq DNA polymerase, 20 pM each of forward and reverse primer and 2µl of template DNA. The reaction was made up to 25 µl by adding nuclease free water. PCR was performed on a thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 94°C for 5 minutes, followed 30 cycles each of denaturation at 94°C for 45 seconds, annealing at various temperature (Table 1, 2) for 45 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes. The PCR product was stored at -20° C.

Table 1: Genus specific primers for identification of bacteria

Organism	Position 5' to 3'	Amplicon Size (bp)	Annealing Temp (°C)	Reference
E. coli	F: ATCAACCGAGATTCCCCCA R: TCACTATCGGTCAGTCAGGAGCAGGAG	232	60	Riffon et al. 2001
Klebsiella	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTC	130	58	Turton et al. 2010
Streptococcus	F: CAGGAAGTGCTGTTACGTTAAAC R: CGTCCCATTTAGGGTTCTTCC	369	55	Jain et al. 2012
Staphylococcus (CP000253.1)	F: CTGTACGCTAGGTGGAGCG (Position: 1092620-1092638) R: TTTTGCAGGATGTCCGCCTT (Position: 1093151-1093132)	532	55	This Study

Table 2: Antibiotic resistance genes primers

Antibiotic gene	**Oligonucleotide sequence (5' to 3')**	**Amplicon Size (bp)**	**Annealing Temp (°C)**	**Reference**
E. coli* and *Klebsiella				
Gentamicin (*aac(3) – iv*)	F: CTTCAGGATGGCAAGTTGGT R: TCATCTCGTTCTCCGCTCAT	285	60	Momtaz et al. 2012
Streptomycin (*aad*A)	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	527	60	Boerlin et al. 2005
Tetracycline (*tet*B)	F: CCTCAGCTTCTCAACGCGTG R: GCACCTTGCTGATGACTCTT	634	60	Momtaz et al. 2012
E. coli				
² actams (*bla*SHV)	F: TCAGCGAAAAACACCTTG R: TCCCGCAGATAAATCACCA	472	60	Karczmarczyk et al. 2011
Klebsiella				
² Lactams (*bla*SHV)	F: TCGCCTGTGTATTATCTCCC R: CGCAGATAAATCACCACAATG	768	58	Momtaz et al. 2012
Staphylococcus				
Tetracycline (*tet*M)	F: AGT GGA GCG ATT ACA GAA R:CATATGTCCTGGCGTGTCTA	158	55	Duran et al. 2012
Erythromycin (*erm*A)	F: TCTAAAAAGCATGTAAAAGAA R:CTTCGATAGTTTATTAATATTAG	645	55	Arana et al. 2014
Methicillin (*mec*A)	F: CCTAGTAAAGCTCCGGAA R: CTAGTCCATTCGGTCCA	331	55	Duran et al. 2012
² lactams (*bla*Z)	F: ACTTCAACACCTGCTGCTTTC R: TGACCACTTTTATCAGCAACC	173	55	Duran et al. 2012
Streptococcus				
Tetracycline (*tet*M)	F: AGT GGA GCG ATT ACA GAA R: CATATGTCCTGGCGTGTCTA	158	55	Duran et al. 2012
Erythromycin (*erm*B)	F: ATTGGAACAGGTAAAGGGC R: GAACATCTGTGGTATGGCG	442	55	Marimon et al. 2005
² lactams (*bla*Z)	F:ACTTCAACACCTGCTGCTTTC R:TGACCACTTTTATCAGCAACC	173	55	Duran et al. 2012
Macrolides (*mef*A)	F: AGTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	346	55	Arana et al. 2014

RESULTS

Isolation of organisms from milk

A total of 102 milk samples from mastitic animals (clinical and subclinical) were tested for the presence of bacterial growth and 50 samples yielded bacterial growth. Of these a total of 43 *Staphylococcus* isolates, 15 *Streptococcus*, 26 *E. coli* and 19 *Klebsiella* were isolated.

Antibiotic sensitivity test

Antibiotic sensitivity test was performed on all the isolates. The antibiotic sensitivity test for *E. coli* revealed that the resistance to ² -lactam was observed in 96.10% of the isolates (25 out of 26), to streptomycin in 80.76% (21 out of 26), to tetracycline in 42.30% (11 out of 26) while to that of gentamicin in 26.92% of the isolates (Table 3).

Antibiotic sensitivity test for *Klebsiella* revealed that the resistance to gentamicin, ² -lactam, streptomycin and tetracycline was 100%, resistance to penicillin was 68.42%, to streptomycin was 47.63% and resistance to the tetracycline and gentamicin was 26.3% (Table 3).

Antibiotic Sensitivity test for *Staphylococcus* isolates revealed that resistance to penicillin was observed in 95.34% (41 out of 43 isolates), to methicillin in 76.74% (33 out of 43), to erythromycin in 55.81% (24 out of 43) and to tetracycline in 51.16% (22 out of 43) isolates (Table 3).

Table 3: Antibiotic resistance and antibiotic resistance genes in different organisms

Organism (No. of isolates)	Antibiotic (Resistant Genes)	Antibiotic resistance and resistance gene (%)
Staphylococcus (43)	Tetracycline (tetM)	22 (51.16)
	Erythromycin (ermA)	24 (55.81)
	Methicillin (mecA)	33 (76.74
	² -lactams (Penicillin) (blaZ)	41(95.34)
Streptococcus (15)	Tetracycline (tetM)	8 (53.33)
	Erythromycin (ermB)	10 (66.67)
	² -lactams (Penicillin) (blaZ)	15 (100)
	Macrolides (Azithromycin) (mefA)	12 (80)
E. coli (26)	Gentamicin (aac(3)-iv)	7 (26.92)
	² -lactams(Penicillin) (blaSHV)	25 (96.10)
	Streptomycin (aadA)	21 (80.76)
	Tetracycline (tetB)	11 (42.30)
Klebsiella (19)	Gentamicin (aac(3)-iv)	5 (26.31)
	² -lactams (Penicillin) (blaSHV)	19 (100)
	Streptomycin (aadA)	13 (68.42)
	Tetracycline (tetB)	9 (47.36)

Antibiotic Sensitivity test for *Streptococcus* isolates observed 100% resistance to the penicillin, 80% (12 out of 15) to macrolides *i.e.* azithromycin and 66.67% (10 out of 15) to the erythromycin while only 53.33% (8 out of 15) to the tetracycline (Table 3).

PCR for genus and antibiotic resistance genes identification

All the isolates when subjected to individual PCR revealed presence of 232 bp product confirming it as *E. coli*, 130 bp confirming it as *Klebsiella*, 369 bp confirming it as *Streptococcus* and 532 bp confirming it as *Staphyloccoccus* (Table 1).

All the *E. coli* isolates were also tested for the presence of resistance genes *viz.*, *aac(3)-iv*, *bla*SHV, *aad*A and *tet*B via PCR. It was observed that ² -lactam resistance gene *bla*SHV was detected in 96.10%, streptomycin resistance gene *aad*A in 80.76%, *tet*B resistance gene in 42.30% and *aac(3)-iv* in 26.92% isolates which is in concordance with the results of antibiotic

resistance data obtained by antibiotic sensitivity test.

All the *Klebsiella* isolates were tested for the presence of resistance genes revealed that *aac(3)-iv* was found in 26.31% of the isolates, *bla*SHV was found in 100% isolates, *aad*A in 68.42% of the isolates and *tet*B in 47.36% of the isolates. Thus, there was variation in the presence of antibiotic resistance and amplification of antibiotic resistance genes which could be attributed to the presence of various antibiotic resistance genes controlling the resistance mechanism (Table 3).

All the *Staphylococcus* isolates were tested for the presence of resistance genes *viz.*, *tet*M, *erm*A, *mec*A and *blaZ* revealed that ² -lactam resistance genes was detected in 95.34%, *mec*A in 76.74%, *erm*A in 55.81% and *tet*M in 51.16% of the isolates which is in concordance with the results of antibiotic resistance data obtained by antibiotic sensitivity test (Table 3).

All the *Streptococcus* isolates were tested for the presence of resistance genes *viz.*, *tet*M for tetracycline, *erm*B for erythromycin, *bla*Z for ² -lactam and *mef*A for macrolides revealed that *blaZ* was detected in 100%, *mef* A gene in 80%, *erm* B in 66.67% and *tet*B in 53.33% of the isolates which is in concordance with the results of antibiotic resistance data obtained by antibiotic sensitivity test (Table 3) (Fig. 1).

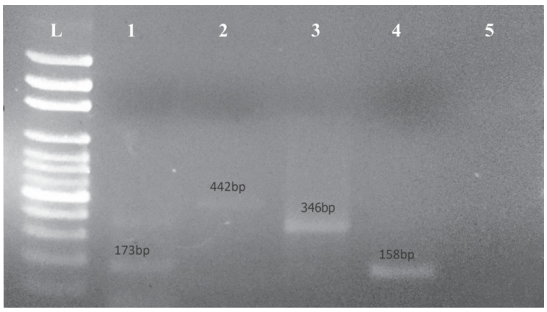

Fig. 1: Agarose gel showing PCR product for antibiotic resistance genes (M 100 bp ladder, Lane 1 *blaZ ,* Lane 2 *erm*B, Lane 3 *mef*A, Lane 4 *tet*M and Lane 5 Negative control

DISCUSSION

In the present study out of a total of 102 mastitic milk samples only 50 yielded bacterial growth comprising 43 *Staphylococcus*, 15 *Streptococcus*, 26 *E. coli* and 19 *Klebsiella*. The isolation of these organisms is a common finding and has been reported by various earlier workers (Ranjan et al. 2010, Sayed et al. 2014, Hawari and Al-Dabbas 2008). All the isolates were subjected to PCR analysis and were found amplifying specfic base pair length product conforming to earlier findings (Riffon et al. 2001; Turton et al. 2010 and Jain et al. 2012).

Further, when all the isolates were subjected to antibiotic sensitivity test comprising erythromycin, methicillin, penicillin, streptomycin, tetracycline, and gentamicin and were also subjected to PCR for identifying presence of antibiotic resistance genes using specific primers, it was observed that there was an absolute correlation between the antibiotic sensitivity results and the presence of genes in the organisms.

The above comparison of genotyping with the phenotyping character of antibiotic resistance has been performed by many earlier workers with success. When correlation between genotyping and phenotyping was studied, it was observed that though there was a correlation between both genotyping and phenotyping but it varied a lot (Strommenger et al. 2003, Duran et al., 2012; Jain et al. 2012 and Gao et al. 2012; Momtaz et al. 2012). In certain studies genotyping was identifying more isolate for antibiotic resistance, whereas in many phenotyping was identifying more. In a study Duran et al. (2012) evaluated the association in staphylococcal isolates and found that methicillin resistance was observed in 17.8% isolates when tested by methicillin disk diffusion method, whereas 27.9% isolates had *mec*A gene indicating more identification using genotyping method. In contrast to these (Yang et al. 2015; Frey et al. 2013; Gao et al. 2012), observed that not all the isolates exhibited antibiotic resistance genes whereas it was exhibited phenotypically. However, there were certain studies which indicated a reasonable correlation between the results of PCR and those of classical resistance testing (Gao et al. 2016). Also, in certain studies it was observed that genotyping was superior to phenotyping (Warsa

et al. 1996, Kresken et al. 2004, Al-Ruaily and Khalil 2011 and Khan et al. 2012). Thus, from the study it could be concluded that as there was an absolute correlation between the genotyping and phenotying results further studies involving more sample size should be performed to establish this finding.

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A Mini-Review on Immunosuppressive Viral Diseases and their Containment Methods at Commercial Poultry Farms

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ABSTRACT

Poultry diseases are one of the major issues of poultry farmers which cause severe economic loss in farming sector all over the world. Many of these diseases are due to viruses which can cause immunosuppression in birds. Also, these agents make birds susceptible to secondary bacterial infection and diseases. Some of these viral agents like Marek's Disease Virus (MDV), Chicken infectious anemia virus (CIAV), Infectious Bursal Disease Virus (IBDV), Reticuloendotheliosis Virus, Lymphoid Leukosis (LL) are silent killers which can cause detrimental and sometimes negative effects on the profitability of poultry farming. All possible attempts should be made to avoid sudden fluctuations in micro-climate of the poultry shed during seasonal changes using improved ventilation strategies, anti-stress nutritional supplementation, optimum stocking densities and strict biosecurity measures. These measures can help in preventing immunosuppression, and maintaining health and welfare of birds.

Keywords: Poultry, Virus, Immunosuppression, Prevention and control

INTRODUCTION

In the past few decades, a significant growth has been achieved in Indian poultry farming sector. Awareness among the poultry farmers, need of nutritious food (protein based), improvement in buying capacity of the people, change in the food habits are few reasons for the boosting change in the face of Indian poultry farming. According to the 19th livestock census, poultry population achieved 12.39% (729.2 million) growth during 2007- 2012 (19th Livestock Census, 2012) in India. Advancement in scientific management practices like, housing, feed formulation, applying avian genetics in breeding programme and diagnosis/prevention of avian diseases resulted in this key achievement. Except for disease control and prevention, all other core management practices can be predicted for the betterment of poultry production. Factors like infectious agents, malnourishment, environmental factors, metabolic disorders, genetic factors, improper management practices etc factually have the negative impact on the economy of the poultry industry.

Infectious agents like bacteria, viruses, fungus can cause diseases in birds with varying severity. These agents may be present in the breeding stock (which can be transferred to next generation), in the farm premises or may be introduced into the farm from a distant place (through the air, water, farm visitors, vehicles, utensils, etc.). They can cause severe morbidity and mortality, leads to an unpredictable production loss in the poultry industry including the loss of important genetic stocks. Moreover, suppression of the immune system by these agents can lead to indirect production losses like growth retardation, poor performance of layers and broilers (low production of egg and meat) and vaccine failure. Some of these microbial agents especially viruses are silent killers which can cause detrimental and sometimes immediate negative effects on the profitability of poultry farming (Bagust, 2013). Through this article, we are trying to give an emphasis on some of the immuno-suppressive viral diseases of chicken which can cause both direct and indirect losses to the poultry industry and their containment.

What is the immune system and immuno-suppression?

Immune-system is the body's defence mechanism to prevent the entry of any kind of foreign particles including infectious agents.

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Immuno-suppression is a status where the immunity is reduced/not at all functioning. Indications of immunosuppression are poor growth and performance, high feed conversion ratio, poor quality of meat and eggs, the incidence of other diseases (e.g. secondary bacterial infections), infertility, high morbidity, and mortality even after proper vaccination etc.

Role of virus in immune suppression

Viruses are agents which can survive only in a living matter otherwise it's an inert particle. Therefore, it has to exploit host immune system to avoid recognition and clearance by the host immune system. On the other hand, the host immune system will respond to virus entry by various immunoregulation mechanism to clear the virus from the host. Viruses evade the host immune system by synthesizing various proteins that mimic important host specific components. For example, viruses like Measles virus down-regulate or inhibit the proliferation of lymphocytes and thus contribute to virus induced immunosuppression (Schnorr et al., 1997). Circovirus like viruses can cause immunosuppression by depletion of lymphocytes (Todd, 2000). Also, virally encoded microRNA can regulate both the virus and host specific transcripts during an actively infected host cells (Fruciet al., 2017).Arai (2019) reports that persistent infection of T and NK cells with Epstein-Barr virus cause immunosuppressive disorders.

Among the avian viruses, Marek's Disease Virus (MDV), Chicken Infectious Anaemia Virus (CIAV), Infectious Bursal Disease Virus (IBDV), Reticuloendotheliosis Virus and Lymphoid Leukosis (LL) have significant role in immunosuppression (Balamurugan and Kataria, 2006). Experimental infection of birds with CIAV, IBDV or MDV resulted in high morbidity and mortality (Rosenberger et al., 1989; Miles et al., 2001). It can directly affect the body's defense mechanism but sometimes it will not produce any clinical disease in affected birds. Although it can cause immunosuppression, the mode of transmission, infection, and occurrence of disease are entirely different. Also, each virus has its own role in immunosuppression like CIAV and MDV affect cell-mediated immunity whereas IBDV affect more the humoral immune response.

Marek's disease (MD)

Marek's disease virus (MDV) is an Alpha-herpesvirus causing tumours of CD4+ T cells (part of the cell-mediated immune system) in viscera, muscle, skin, and lesions in peripheral nerves in chickens. It can cause severe lymphoproliferation and infiltration of mononuclear cells in various parts of the body. Infiltration of lymphoma cells can lead to the impairment of defense mechanism which indirectly affects growth and performance (Adair, 2000). During the first two weeks of birds, it can cause severe depletion of lymphocytes (the major component of the immune system) which leads to marked atrophy of the lymphoid organs namely bursa and thymus. Depending on the virulence of the virus the atrophy can be irreversible or transient.

Two phases of immunosuppression are observed in MD infection. Early immunosuppression associated with cytolytic infection in the lymphoid organs in chickens lacking maternal antibodies against MDV and late immunosuppression that appears later in the pathogenesis and occurs even in chickens bearing antibodies against MDV (Faiz et al., 2017).In earlier stage, immunosuppression is may be due to death of MDV infected cells and in later stage may be due to activation of regulatory T (Treg)-cells. MDV-induced lymphoma cells expressed high levels of TGF-beta. Release of TGF-beta along with PGE2, and some of the soluble factors in MDV-induced tumour cells can inhibit T cell function, and may contribute to MDV-induced immunosuppression (Gurung et al., 2017).

Chickens are the most important natural hosts for MDV but turkeys can be affected experimentally. Current vaccines may not provide sterile immunity and even, it may lead to emergence of increasingly virulent strain of MDV. This may be due to the genetic background of birds, concurrent infection with other immunosuppressive viruses and virulence of MDV strain.

Chicken infectious anaemia (*Blue wing disease*)

Chicken infectious anaemia virus (CIAV) is one of the economically important and re-emerging pathogens of chicken and is ubiquitous in farm premises worldwide (Bulow and Schat, 1997). It may be one of the major culprits causing secondary infections in chickens. It can cause severe disease in young chicks of up to 1-3 weeks of age (Adair, 2000). The virus can infect chickens of all ages but only young chicks may develop clinical signs. The importance of this disease is in young chicks, it impairs the thymus causing immunosuppression which leads to major economic losses in broiler production with poor weight gain and heavy mortality. CIAV can be transmitted vertically as well as horizontally. The infection occurs naturally when breeder flocks, just before or during egg production, with no previous exposure to the virus, become infected. Under these conditions, the infected progeny develop the disease symptoms, including thymus atrophy, haemorrhages and anaemia, from 10-14 days of age. Horizontal transmission is through the contaminated environment like litters, water, utensils etc. Clinical disease may not occur in chicks with high maternal antibody titre but do not prevent infection and transmission of the virus or immunosuppression (Balamurugan and Kataria, 2006). Both clinical and subclinical CIAV infections along with secondary bacterial/viral infections have a significant effect on commercial broiler/layer performance and profitability.

CIAV target erythroid and lymphoid progenitor cells (haemocytoblasts and precursor T lymphocytes and also reticular cells). Its replication in thymocytes in the cortex of the thymus cause destruction of T-lymphocytes and directly cause apoptosis of bone marrow haematopoietic precursors (hemocytoblasts), causing transient severe anaemia and immunosuppression (Balamurugan and Kataria, 2006). Hemocytoblasts are the precursors for red blood cells, thrombocytes, and heterophils, whereas thymocytes are the precursors for $CD4^{+}$ Th lymphocytes and $CD8^{+}$ CTLs. Complete destruction of the hemocytoblasts by CIAV can result in anaemia, haemorrhages and increased susceptibility to bacterial infections, while apoptosis of thymocytes affects Th functions, which are essential for immunoglobulin Y (IgY) and IgA antibody production and CTL functions. Dividing B-cell precursors are generally considered to be refractory to infection (Gimeno and schat, 2018).

The early infection in young chicks may cause aplastic anaemia and older birds may have impaired T lymphocyte and macrophage activities, and loose bactericidal capability. This may lead to the development of secondary infections, including gangrenous dermatitis, coccidiosis, viral or bacterial infections (Hoerr, 2010). There is no specific treatment for the disease and there is need for time to time surveillance of CIAV among the flocks for proper containment of CIA. Live attenuated vaccines are available in other countries but vertical transfer, viral persistence and reversion of viral virulence are the important problems in using these vaccines (Sawant et al., 2015).

Infectious bursal disease (*Gumboro Disease*)

Infectious Bursal Disease Virus (IBDV) is another important viral agent causing immunosuppression among chicken. The first report of IBDV was during 1962 and even after 4 decades of its discovery, IBDV cause severe economic loss in the poultry industry. Among the two serotypes, only serotype one causes clinical disease and immunosuppression in chickens. IBDV can cause severe immunosuppression with impaired antibody response and high susceptibility to secondary diseases particularly when chicks get infected before three weeks of age.

The molecular basis of IBDV induced immunosuppression is the result of multiple interactions between different viral proteins (VP's) and infected B cells (Allan et al., 1972). The major target cells of IBDV serotype 1 strains are lymphoid cells in the Bursa of Fabricius. It replicates mainly in IgM positive B lymphocytes, especially in the bursa, and also replicate in macrophages in the bursa. Infection in the Bursa of Fabricius may lead to depletion of B lymphocytes. Apoptosis of infected cell is induced by VP5 and VP2 proteins (Gimeno and schat, 2018). Addition of type I IFN in IBDV infected cells promotes apoptosis which can be a mechanism of virus clearance from the site of infection. The dsRNA of IBDV may up-regulate PKR, TNF±, and NF kappa B thereby inducing

apoptosis of infected cells. The negative side of this apoptic clearance of virus is the depletion of B cells which may leads to immunosuppression (Cubas-Gaona, et al., 2018). IBDV are highly resistant to physical and chemical agents which leads its persistence in the outside environment, particularly on contaminated farms, in spite of disinfection and decontamination. Therefore, strict hygienic measures along with medical prophylaxis are must for the prevention of persistence of this hardy virus in the farm premises. Vaccines (Attenuated live vaccines and inactivated) are available and success of vaccination is depending on the strains used in the vaccination program. The pathotype and antigenic variant of the virus must be considered while implementing the vaccination schedule (van den Berg et al., 2000).

Avian leukosis

Avian leukosis virus (ALV) cause proliferation of haematopoietic cells which leads to leukaemia-like proliferative diseases among birds. It causes neoplastic change in the lymphopoietic, erythropoietic and myelopoietic system. Among this, lymphoid leukosis has been the most common form of the leukosis/sarcoma group of diseases seen in chicken flocks. It causes transformation of the intact bursa, often as early as 6-8 weeks after infection. Death rarely occurs before 14 weeks of age and is more frequent around the time of sexual maturity. The disease can be prevented, even up to 5 months of age, by treatments that destroy the bursa (Cheville, et al., 1978). Among various Avian leukosis virus, subgroup J (ALV-J) cause neoplasia and immunosuppression in chickens and also an interruption of the switch of antibody IgM to IgG in B cells infected with ALV occurs. Natural infection occurs in chicken whereas experimental infection/tumours can be produced in other species of birds. Subclinical infection may affect performance traits, including egg production and quality of birds. No specific treatments or vaccines are available for control of the leukosis but eradication of exogenous ALV from breeding stock to produce infection-free parent breeds, together with hygiene measures aimed at preventing re-infection or limiting spread of infection among poultry flocks (Payne and Venugopal, 2000).

Effect of virus interaction on immunosuppression

Co-infection of one or more virus may aggravate the immunosuppression and further disease progress among birds. CIAV infection along with IBDV may cause severe anaemia (Rosenberger and Cloud, 1989), decreased weight gain and gangrenous dermatitis in chicken. MD and CIAV may cause early chick mortality syndrome. The production of CTL can be reduced due to CIAV infection which may influence the protection by MD vaccine. IBDV infection along with MD reduced the incidence of MD compared to birds infected with only MDV (Gimeno and Schat, 2018).

Prevention and control

“Prevention is better than cure”- surely prevention is most important while dealing with infections and diseases in poultry. To achieve good performance, the quality of micro, as well as the macro environment, should be well maintained up to the maximum possible level. This helps in maintaining the bird health, welfare and prevention of immunosuppression. Stress is one of the important factors to be considered and it should be minimised to the lowest possible level (along with poor ventilation, cool ambient temperature and high humidity, immunosuppression also contributes stress). Careful selection of healthy birds especially from farms or hatchery with the history of minimum disease occurrence or outbreaks is desirable. Quarantine of the new flocks should be strictly followed. High level of hygiene in farm and its surrounding can reduce the microbial load. Control the personal movement in and out of the farm to control the transmission of an infectious agent from one place to another.

Timely vaccination against important microbial agents (like virus/bacteria) is another important factor to prevent infection and disease. But vaccination alone cannot be considered as an alternative to the good management practice, to the biosecurity measures and to the control policies for the prevention of any infections or the diseases. Strategies like passive and active immunization of birds have usually followed in poultry industries. In passive immunization parent stock is vaccinated to infectious agents

and immunity developed is transferred from parent to next generation, so that early protection from field challenge can be expected. Active immunity is achieved by immunizing birds with a vaccine *in ovo* or after hatching and further by boosters at particular time intervals. It should be bear in mind that no vaccine could provide 100 percent protection to the vaccinated birds/flocks under field condition as there are many factors which influences the efficiency of a vaccine.

Factors to be considered while implementing a vaccination programme

- Type of farming - Commercial or rural type of poultry farming
- Species of birds and density of each species
- Status of disease occurrence and prevalence in the locality
- Availability of vaccine and veterinary aid
- Cost of implementation
- Herd immunity – the positive effect of vaccination in a flock/area where possibility of getting infection to an unvaccinated bird is reduced when it is part of a vaccinated flock

The vaccination programme may include routine vaccination, emergency vaccination and preventive vaccination (Marangon and Busani, 2006). Routine vaccination can be applied in an endemic area against prevailing infection. It can lower the mortality and production loss in long run farms. Emergency vaccination is important when a new infection or disease is introduced in the farm/area which can spread the infection rapidly. Preventive vaccination is generally carried out for the prevention of poultry diseases that have a clear impact on the industry like risk of introduction and further spread (Bouma et al., 2004). Prophylactic vaccination together with the application of effective biosecurity measures can provide maximum protection during an outbreak.

Conclusion

Disease outbreak and mass mortality are common in many poultry farms, even following strict management practices, good hygiene, healthy foods etc. Is this indicating if there is some loophole inmanagement practices and/or efficiency of current vaccines may be incomplete? Changing climate, global warming along with high demand for animal protein and intensive system of poultry raring may help infectious agents to mutate and emerge as new variant/strain. Subclinical nature of some of the viruses and suppression of immune system is ultimately making poultry population vulnerable to many other pathogens. Also, incorrect disease diagnosis and unbalanced use of antimicrobials help pathogens to gain an upper hand in poultry health. The economic loss due to these outbreaks may be enormous. Direct loss is from drastic fall in production and indirect loss from post-outbreak control measures like control of infection, screening of flocks, and implementation of new prophylactic measures including development of newer vaccines. Understanding more about immunosuppression may help the development of novel vaccines. The effectiveness of vaccination is depending on nutrition which contributes strong immunity among vaccinated flocks. No geographical location is completely safe from invading infectious/pathogenic organisms. Strict hygiene and biosecurity measures along with appropriate vaccination strategies should be implemented to contain the disease occurrence. To control immunosuppression, its dire necessary to reduce stress level in poultry by reducing exposure to these silent killers through biosecurity measures and increasing disease resistance through selecting genetically disease resistant germplasms. Also, Immunomodulators and adjuvants are promising future candidate for counter acting the immunosuppression.

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Comparison of Nested and Real-Time PCR for Detection of Latent Equine Herpesvirus 1 Infection

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ABSTRACT

Equine herpesvirus 1 (EHV1) is one of the most important equine viral pathogen. Following acute infection, recovered animals develop lifelong latent infection, with periodic reactivation. The objective of this study was to compare nested and real-time PCR targeting glycoprotein B (gB) gene for detection of latent EHV1 infection. The real-time PCR (gB-qPCR) assays detected 41 gene copies/ reaction while nested PCR (gB-nPCR) assays could detect 4100 gene copies/ reaction. For assessing diagnostic sensitivity of both the assays, an abortion outbreak was followed for 6 months. After 6 months, none of the aborting (24) mares was shedding virus in nasal and vaginal swabs. However, latent EHV1 infection was detected in 7 and 15 mares by nested and real-time PCR respectively, by demonstration of viral DNA in peripheral blood mono-nuclear cells (PBMCs) in the absence of detectable late structural gene mRNA using gB-based real-time PCR. The sensitivity of gB-nPCR was only 46.66% as compared to gB-qPCR for detection of EHV1 latent infection. The real-time PCR is a sensitive and specific assay for ante-mortem detection of EHV1 latency in equine population.

Key words: Equine herpesvirus 1, latency, PCR, real-time PCR, sensitivity

INTRODUCTION

EHV1 is an important, ubiquitous equine viral pathogen that causes significant economic losses to the equine industry and produces well documented syndromes of respiratory disease, abortion, neonatal foal death and occasionally myeloencephalopathy (Brosnahan and Osterrieder, 2009; Liu et al., 2017). EHV1 is an enveloped, double-stranded DNA virus belonging to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae*, order *Herpesvirales* (Davison et al., 2009).

As with other ±-herpesviruses, EHV1 produces life-long latent infection in the neurons within the trigeminal ganglia, lymphoid tissues and/or lymphocytes in lymphoreticular tissues in over 50% cases, following natural infection (Foote et al., 2006; Allen et al., 2008; Pusterla et al., 2012; Walter et al., 2013). During EHV1 latency, viral genome is present in infected cells but only a limited part undergoes transcription, giving rise to latency associated transcripts (LATs) (Allen et al., 2008; Pusterla et al., 2012). Two LAT sequences have been identified in latent EHV1 infections, LAT1, which is located on genome strand complementary to 3′ of ORF63 and LAT2 present at genome strand complementary to 3′ of ORF64 (Baxi et al., 1995). The LAT sequence overlapping and antisense to ORF64 is more consistently reported in EHV1 latency (Borchers et al., 1999; Pusterla et al., 2009).

Ante-mortem diagnosis of latent EHV infection is a major challenge by the currently available diagnostic methods. The latency can be detected by showing the expression of latency associated transcripts (LATs). During latency, EHV1 DNA is present in selected tissues but no transcription of late genes take place. Therefore, latent infection is confirmed when tissue samples is found PCR positive for the late structural glycoprotein B (gB) DNA in the absence of detectable gB mRNA in the samples. The PCR and real-time PCR assays targeting gB, have become important tool for detecting both acute and latent EHV1 infections (Carvalho et al., 2000; Allen et al., 2008; Pusterla

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et al., 2009). This study compares PCR and real-time PCR targeting gB for detection of EHV1 latency.

MATERIALS AND METHODS

Viruses

Reference EHV1 strain V592 procured from Animal Health Trust, Kentford, Newmarket, United Kingdom and available at NRCE was used as positive control. The virus stock was prepared by inoculating RK-13 cells and incubating till 80% cells showed cytopathic effects. The virus was recovered from infected cells by three freeze-thaw cycles, followed by centrifugation at 10000 × g for 20 min to remove cellular debris. The virus was titrated using RK-13 cells in 96-well tissue culture plates (Corning, NY, USA) as 50% tissue culture infected doses ($TCID_{50}$) by Reed and Muench (1938). The titrated virus was aliquoted and stored at -80°C till further use.

Equine herpesvirus 4 (EHV4/Hisar) and equine adenovirus (EAdV/H9), maintained at ICAR-NRCE, Hisar were used to test the specificity of the assays.

Sample collection

An outbreak of abortions occurred in mares at equine farms in Hisar (Haryana) during January-May 2014. The cervico-vaginal swabs, Nasal swab and blood (in EDTA) from all aborted mares were collected on day of abortion and transported on ice to the laboratory. Vaginal swab, nasal swab and blood samples (in EDTA) from aborted mares were again collected in October 2014.

Peripheral blood mononuclear cells (PBMCs) were separated by overlaying blood over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifugation at 400 x g. After two washings with PBS, PBMCs were suspended in PBS to make $2x10^6$ cells/ml. Nasal and vaginal swabs were collected in 2 ml transportation medium (EMEM supplemented with 2% FBS and 1% antibiotic-antimycotic solution). Swabs were squeezed and suspension was centrifuged at 1,700 x g for 15 min and supernatant stored at -80°C until further processing.

Nucleic acid extraction

DNA was isolated taking 200 µl of PBMCs or tissue homogenate using DNeasy Blood & Tissue DNA extraction kit (Qiagen, India) according to the manufacturer's recommendations to elute in 40 µl of elution buffer provided along with the kit. Nucleic acid concentration was measured using the Eppendorf BioPhotometer plus (Eppendorf, Germany). The DNA copy numbers were calculated using online available ThermoFischer DNA copy number and dilution calculator (https://www.thermofisher.com/sg/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html)

Total RNA was extracted from 250 µl each of PBMCs, using TRI reagent (Sigma-Aldrich, USA). RNA was treated with DNase for 30 min at 37°C to remove genomic DNA and RNA purity was checked by PCR amplification of housekeeping gene equine glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Virus isolation and Identification

Swabs collected from aborted mares were processed for virus isolation. Briefly, samples were inoculated onto 25 cm^2 RK-13 cell culture flasks at 37°C under 5% CO_2 for 5 days and observed daily for development of cytopathic effects. DNA was isolated from cell culture lysate was tested for EHV1 virus by nested PCR (OIE-nPCR) using primers (Table 1) following the method of Borchers et al. (1993). Three blind passages were made before declaring the samples negative.

Detection of latency by nested RT-PCR

DNA-free RNA was used for cDNA synthesis using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific, India) and stored at -70°C. A nested RT-PCR (gB-nPCR) for detection of EHV1 latency was standardized using primers (Table 2) to amplify a 188 bp fragment of *gB* (Kirisawa et al., 1993) using peqSTAR Thermocycler (PEQLAB, Germany). The reaction

mix (25 µl) for outer and nested PCR consisted of 12.5 µl of 2X Top Taq Master Mix (QIAGEN, Hilden, Germany), 1 µl each of forward and reverse primer (500 nM each primer), 5.5 µl of nuclease-free water, and 5 µl of DNA template. The PCR components were mixed properly and set with the amplification conditions for both outer and nested PCR as follows: initial denaturation step at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 7 min.

Lower limit of detection for this assay was determined by taking serial 10-fold dilutions of EHV1/V592 DNA.

Table 1. Oligonuclotide primers used in the present study

Assay	Primer Name	Primer Sequence (5'→3')	Location (nt)	Amplicon size (bp)	Reference
gB-qPCR	gB-qPCR/for	TATACTCGCTGAGGATGGAGAC	61751-61775	90	Pusterla et al., 2009
	gB-qPCR/rev	TTTTTGGGGCAAGTTCTAGGTGGTT	61840-61819		
	gB-qPCR/probe	6FAM-ACACCTGCCCACCGCCTACCG-BHQ1	61777-61797		
OIE-nPCR	OIE-nPCR/outer-for	TCTACCCCTACGACTCCTTC	62281-62300	1473	Borchers and Slater, 1993
	OIE-nPCR/outer-rev	ACGCTGTCGATGTCGTAAAACCTGAGAG	63754-63727		
gB-nPCR	gB-nPCR/outer-for	CTTGTGAGATCTAACCGCAC	62841-62860	1180	Kirisawa et al., 1993
	gB-nPCR/outer-rev	GGGTATAGAGCTTTCATGGG	64021-64002		

Detection of latency by real-time RT-PCR

The relative quantification of *gB* gene was done by quantitative PCR (gB-qPCR) assay using primers and probe (Table 2). For real-time PCR assays, 25 µl reaction mix consisted of 12.5 µl of 2X Quantitect Multiplex Master Mix (QIAGEN, Hilden, Germany), 1 µl each of forward and reverse primer (400 nM each primer), 1 µl of probe (80 nM), 4.5 µl of nuclease-free water, and 5 µl of DNA template. Each sample was tested in triplicate. The amplification was done using CFX96™ Real-Time System (Bio Rad, Singapore) with an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s and annealing/amplification at 60°C for 60s.

Standard curve was generated with serial 10-fold dilutions of EHV1/V592 DNA template. The DNA copy numbers were calculated using online available Thermo Fischer DNA copy number and dilution calculator (https://www.thermofisher.com/sg/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html).

Nucleic acid from EHV1/V592; EHV4/Hisar and EAdV/H9 were used as template to determine the specificity of the nested and real-time PCR assays.

RESULTS

Standardization of nested and real-time PCR

In order to diagnose latent EHV1 infection, nested (gB-nPCR) and real-time PCR (gB-qPCR) targeting *gB* were standardized. EHV1 gB-nPCR was developed that amplified 188 bp of

glycoprotein B. The assay sensitivity was determined to be 1340 fg or 4.1 x 10^3 gene copies. For gB-qPCR standard curve was generated with serial 10-fold dilutions of EHV1/V592 DNA template**.** From the standard curve, the slope was calculated as -3.658 and the correlation coefficient (R^2) was found out to be 0.999. By using obtained Cq value and slope, the amplification efficiency was calculated to be 87.7% (Fig. 1). The sensitivity of the real-time assay was found to be 13.4 fg or 41 copies.

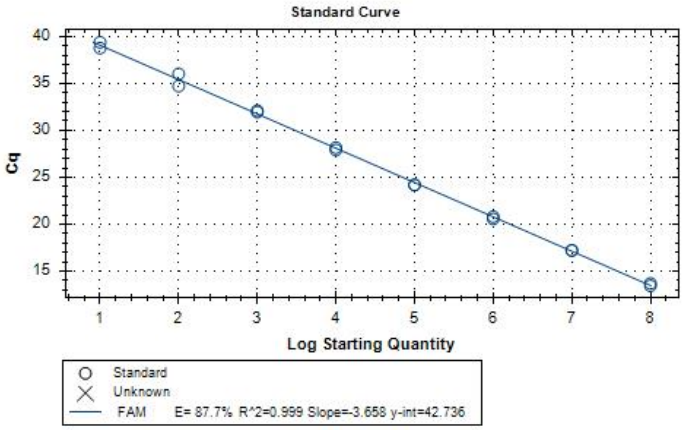

Fig. 1: Analytical sensitivity of gB-qPCR. Standard curve was obtained using 10-fold dilutions of EHV1/V592 DNA with a slope of -3.658, correlation coefficient (R2) of 0.999 and efficiency of 87.7%.

Both the assay was found to be specific for EHV1 and did not react with other equine DNA viruses *viz*. EHV4/Hisar and EAdV/H9.

Detection of latent EHV1 infection

During January 2014 to May 2014, 40 mares aborted in last trimester of pregnancy. EHV1 was detected in vaginal swabs of 24 mares by OIE-nPCR and further confirmed by virus isolation from 15 mares (Fig. 2). After 6 months of abortion, none of the aborted mares with laboratory confirmed EHV1 infection (n=24) tested positive for virus shedding in nasal and vaginal swabs by gB-nPCR and gB-qPCR. However, Viral DNA was detected in PBMC gDNA in 7 mares by gB-nPCR (Fig. 3) and 15 mares by gB-qPCR with $1.38x10^4$-$7.48x10^5$ copies/million cells, while, PBMC cDNA tested negative in all mares by gB-nPCR and gB-qPCR, indicating non-transcription of EHV1 late structural gene.

Relative sensitivity and specificity of the gB-nPCR assay for EHV1 latency was estimated by comparing with that of gB-qPCR for detection of EHV1 *gB*. The sensitivity and specificity of gB-nPCR were 46.66% and 100%, respectively (Table 2).

Table 2: Sensitivity and specificity of nested PCR for detection of EHV1 latency

Screening test	Results	gB-nPCR		Sensitivity (%)	Specificity (%)
		Positive	Negative		
gB-qPCR	Positive	7	8	46.66	100
	Negative	0	9		

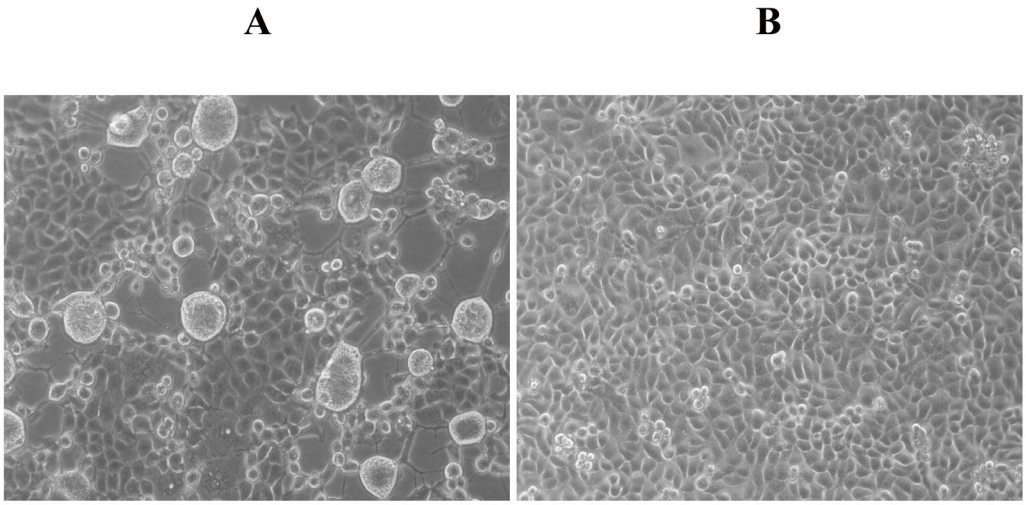

Fig. 2: Cytopathic effects produced by EHV1 isolated from vaginal swab of mare (#1409) in RK13 cells (A), non-infected control cells (B). Magnification 200X

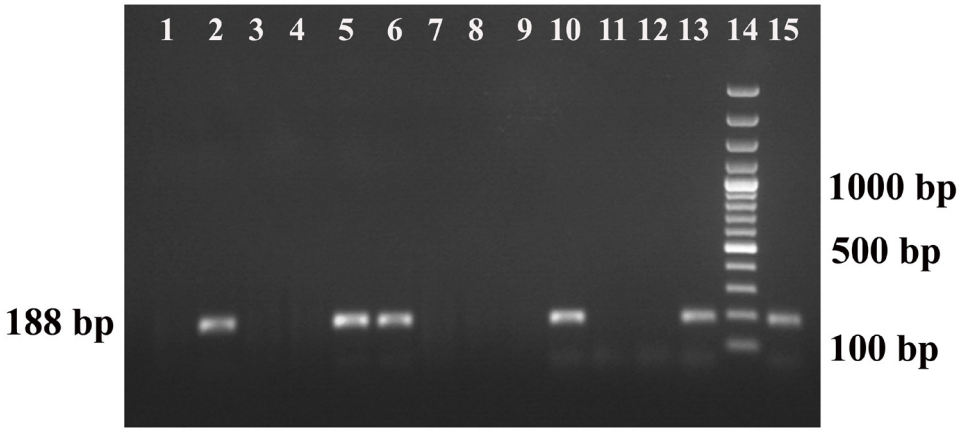

Fig. 3: Detection of EHV1 in PBMCs of mares 6 month after abortion by gB-nPCR. Lane 1: non-template control; Lane 2: mare #1401; Lane 3: #1402; Lane 4: #1403; Lane 5: #1404; Lane 6: #1405; Lane 7: #1406; Lane 8: #1407; Lane 9: #1408; Lane 10: #1409; Lane 11: #1410; Lane 12: #1411; Lane 13: #1412; Lane 14: GeneRuler 100 bp plus DNA Ladder (Thermo Scientific, India); Lane15: EHV1/V592 as positive control. gDNA for gB but not transcripts were detected in mares #1401, 1404, #1405, #1409 and #1412

DISCUSSION

EHV1 is the most important pathogen affecting the horses globally (Davison et al., 2009; Brosnahan and Osterrieder, 2009). It is estimated that 80-90% of horses get exposed to EHV1 infection by 2 years of age followed by establishment of life-long latent infection, expressing latency associated transcripts (Borchers et al., 1999; Pusterla et al., 2012). EHV1 establishes latency/persistent infection in neurological tissues, lymphoid tissues and peripheral blood leukocytes (PBL) (Ma et al., 2010). EHV1 latent infection can be diagnosed if there is presence of late structural gene (*gB*) DNA in absence of its mRNA. The primary aim

of this study was to compare sensitivities and specificities of nested and real time PCR targeting gB for detection of latent EHV1 infection. For specific detection of latent EHV1 infection targeting late structural gene (*gB*), gB-nPCR and gB-qPCR assays were standardized in the present study. The detection limit of gB-qPCR was 100 times higher than that of gB-nPCR, similar to observation of Marusic Vrsalovic et al. (2007). The amplification efficiency of gB-qPCR was similar to that reported previously (Pusterla et al., 2009). The assay developed by Hussey et al. (2006) had a detection limit of 6 copy of gene, which is slightly better than our assay (41 copies).

Until now, there have been no data on the evaluation of two PCR methods (nPCR, and qPCR) against the same target gene specific for EHV1 *gB*. For the reference strains used here, the specificities of the two kinds of PCR assays were very good. No positive results were detected in the nPCR and qPCR assays. In our study, the detection sensitivities of both kinds of PCR assays

differed. The lower limit of detection of the nPCR assay was 4100 copies of gB gene. However, the lower limit of detection of the qPCR assay was 41 copies, and it had the best detection sensitivity. If we used cutoff values of 35 Cq as positive results, the results of the q-PCR assay using blood and tissue samples showed 100% sensitivity and specificity. Therefore, our study suggests that appropriate use of the qPCR assay using blood and tissues is useful for the rapid diagnosis of latent EHV1 infection.

The confirmation of latent infection by ante-mortem examination is very difficult from samples such as blood. For the nPCR assays, it takes at least 6 h to detect the EHV1 gB-specific gene, and the sensitivity of the nPCR assay is less (46.66%). The qPCR assay can give results within 2 h from genomic DNA extraction to data analysis after amplification (start to finish) andwas found to be 100% sensitive in our study. It is very useful for establishing an early diagnosis, has the potential for automation for high-throughput analysis, and yields quantitative information.

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Conflict Of Interest

All authors declare that there is no conflict of interest.

Ethical Approval

This article does not contain any experiment with human participants or animals performed by any of the authors.

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Expression of Cathelicidins in Bovine Tissues

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ABSTRACT

Anti microbial proteins (AMPs) are host defense molecules that constitute an important part of the innate immunity in animals. This study was carried out to investigate the expression of some related AMPs belonging to cathelicidins family; namely cathelicidins 1, cathelicidins 2, cathelicidins 3 and cathelicidins 4 in seven milk samples with varying somatic cell counts, udder and liver tissues of Jersey Red Sindhi crossbred cows. For this, milk samples were collected and evaluated for somatic cell counts. Liver and udder tissue were also collected for studying the expression of these AMPs. The gDNA was isolated from liver tissues. Pairs of primers viz CathL1, CathL2, CathL3 and CathL4 were designed and tested initially on gDNA. Total RNA was isolated from the tissues and the milk samples followed by cDNAs preparation. Expression of cathelicidins coding genes vizcathelicidins1, cathelicidins 2 and cathelicidins 3 was found in liver tissue but not of cathelicidins 4 gene. Expression of cathelicidins 2 gene was found in udder tissue but not of cathelicidins 1, cathelicidins 3 and cathelicidins 4 genes, respectively. Expression of cathelicidins1, cathelicidins 2, cathelicidins 3 and cathelicidins 4 genes, respectively was not found in the selected milk samples of Jersey Red Sindhi crossbred cows.

Key words: Anti microbial proteins, cathelicidins, gene expression, Jersey Red Sindhi crossbred cow

INTRODUCTION

Antimicrobial proteins (AMPs) play an important role in defence against infectious diseases caused by various pathogenic microorganisms. AMPs, also called "host defense peptides" may be present in a number of systemic organs and variety of tissues. These protein or peptide molecules are generally between 12 and 50 amino acids and have been demonstrated to kill Gram negative and Gram positive bacteria, enveloped viruses and fungi(Reddy et al., 2004). Due to their bactericidal and bacteriostatic properties, the study of the presence of AMPs in bovine milk and other tissues is a highly coveted area of interest as these AMPs could be the source of innate immunity to animals exposed to infectious agents. Cathelicidins are one such potential group of AMPs. These are small cationic antimicrobial peptides found in humans and other species, including farm animals (cattle, horses, pigs, sheep, goats, chickens, rabbits and in some species of fish) (Brogdenet al*., 2003*). These peptides show a broad spectrum of antimicrobial activity against bacteria, enveloped viruses and fungi (Ulmet al*., 2012)*. Apart from exerting direct antimicrobial effects, cathelicidins can also trigger specific defense responses in the host. Their roles in various patho-physiological conditions have been studied in mice and humans, but there are limited information about their expression sites and activities in livestock. Cathelicidin-related antimicrobial peptides are a family of polypeptides found in lysosomes and macrophages of polymorphonuclear (PMN) cells. Members of the cathelicidin family of antimicrobial polypeptides are characterized by a highly conserved region (cathelin domain) and a highly variable cathelicidin peptide domain (Zanetti, 2004). Some of the bovine cathelicidin family members whose expression is investigated in this study are cathelicidin1 (or BAC1), cathelicidin 2 (or BAC5) cathelicidin 3 (or BAC7) and cathelicidin 4 (or indolicidin).The expression of cathelicidin AMPs has not been studied so far in the bovines reared commonly in Himachal Pradesh, India. Such a study will enable us to know the status of these AMPs in locally reared cows. Therefore, this study is envisaged with the objectives of determining the expression of

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cathelicidins in bovine milk comprising of varying counts of somatic cells, udder and liver tissues.

MATERIALS AND METHODS

Collection of milk and tissue samples of cows

The present study was conducted on seven fresh milk samples collected in diethylpyrocarbonate (DEPC) treated, autoclaved 50 ml centrifuge tubes from the lactating Jersey Red Sindhi cross bred cows housed at livestock farm of Dr. G. C. Negi College of Veterinary and Animal Sciences, CSK Himachal Pradesh Agriculture University, Palampur, India. Liver and udder tissue samples, two each were collected in autoclaved plastic vials within half an hour of death of the cows and transported in cryocan to laboratory.

Somatic cell count

Total somatic cell count was done as per the modified technique of leukocyte count described by Prescott and Breed (1910) and classification of animals based on mastitis was done as per Malinowski (2001).

Isolation of genomic DNA (gDNA)

The gDNA of liver tissue sample of Jersey Red Sindhi crossbred cow was isolated as per phenol-chloroform-isoamyl alcohol method (Strauss, 2001) and was assesed by electrophoresing in 1.2 % agarose gel run in tris borate EDTA buffer.

Selection of pairs of primers

Pairs of cathelicidin primers *viz*: CathL1, CathL2, CathL3 and CathL4, were designed complimentary to cathelicidin1, cathelicidin 2, cathelicidin 3 and cathelicidin 4 genes, respectively**.** Structural organization of four pairs of primers used in this study, each containing a forward (FP) and reverses (RP) primer across the four cathelicidin genes is depicted in Fig. 1. Selection of a pair of TATA-box-binding protein (TbP) primer as designed by Lisowski et al. (2008) was done based upon the expression of the house keeping TATA-box-binding protein (TBP) gene. The nucleotide sequences of forward and reverse primers complementary to the four cathelicidin genes: cathelicidin1, cathelicidin 2, cathelicidin 3 and cathelicidin 4 and TBP gene are described in Table 1.

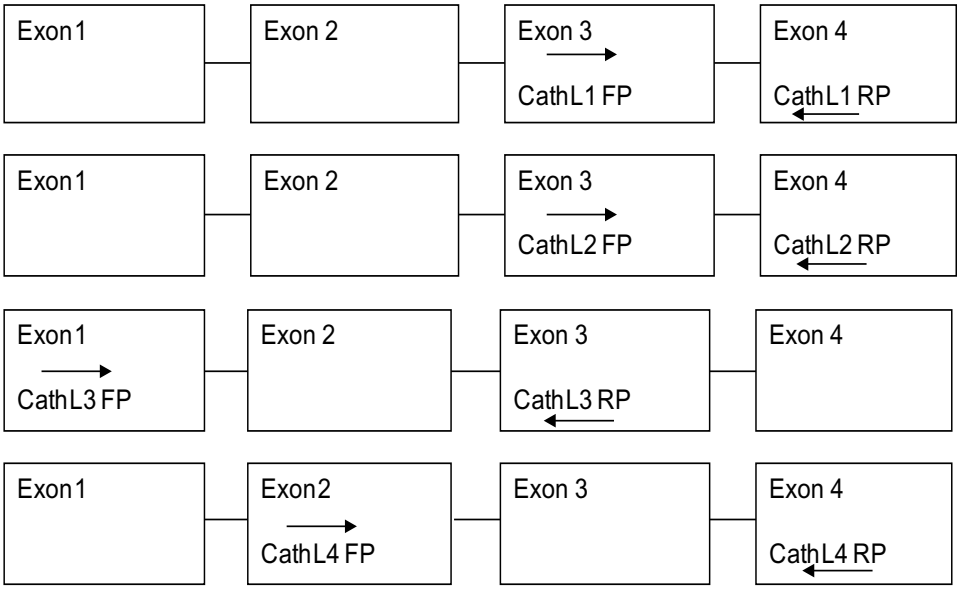


Fig 1: Structural organization of four pairs of primers (each having a forward and reverse primer) used across four cathelicidin genes of cattle.

Table 1: Primer sequences of CATHL and TBP genes

Primer	Gene amplified	Primer Sequences (5'-3')
CathL1 FP	Cathelicidin1	GGTCAGGGGTAACTTCGACA
CathL1 RP		CCTTAGGACTCTGCTGGCTT
CathL2 FP	Cathelicidin2	GTGAAACAGTGTGTGGGGAC
CathL2 RP		GAAACGGTCCTAAGGGTGGA
CathL3 FP	Cathelicidin3	TGCTGTGGATCGCATCAATG
CathL3 RP		GTCCCCACACACTGTTTCAC
CathL4 FP	Cathelicidin 4	CTCGAAAGCCTGTGAGCTTC
CathL4 RP		TAGGATGACACTCTGGAGCTCA
TbP FP	TATA-box-binding	ACAACAGCCTCCCACCCCTATGC
TbP RP	protein gene	GTGGAGTCAGTCCTGTGCCGTAA

PCR on gDNA

Bovine gDNA isolated from liver tissue sample of Jersey Red Sindhi crossbred cow was used as a template in the PCR reactions with various sets of primers (Table 1) in a final reaction volume of 25 µl in Eppendorf Master cycler PCR machine**.** Pairs of primers were used at a final concentration of 0.4 µM/ µl in PCR reactions to produce amplicons, respectively. A pair of TbP primer was used as an internal control. PCR amplification of TBP gene was performed as per Lisowski et al. (2008).

Isolation of total RNA from liver and udder tissue samples

Total RNA from liver and udder tissues of the cows were isolated using RNeasy® Mini kit, Part 1 (Qiagen).

Isolation of total RNA from milk samples

A number of fresh milk samples (about 40) collected from the four quarters of the cows were mixed and a sub sample of 50 ml was used for total RNA isolation. Milk somatic cells were pelleted by adding 50 µl of 0.5 ethylene diamine tetra acetic acid (EDTA) to 50 ml of fresh milk and centrifuged at 218 g at 4°C for 10 minutes. The pellet of cells were washed with 10 ml phosphate buffered saline (PBS), at pH 7.2 and 0.5 mM EDTA and filtered through sterile cheese cloth to remove any debris. Milk samples were centrifuged again at 218 g at 4°C for 10 minutes. The supernatants were decanted and the total RNA was isolated from milk cell pellets by RNeasy® Mini kit, Part 2 (Qiagen). Total RNA isolated from liver and udder tissue and selected milk samples were assesed by electrophoresing in 1.2 % (w/v) agarose gel in tris borate EDTA buffer and concentration was measured using BioDrop, UK.

Preparation of complementary DNA (cDNA) and amplification of cathelicidin transcripts

Total RNA (100 ng each) isolated from different samples was used as a template in RT-PCR reaction that was carried out in a final reaction volume of 20 µl in Eppendorf Master cycler PCR machine**.** Quantity and concentration of other components used in RT-PCR comprised of 2.0 µl of 10X RT buffer (Invitrogen), 2.0 µl of dNTPs (5 mM) (Invitrogen), 1.0 µl of oligo dT primer (10 µM) (Invitrogen), 1.0 µl of Omniscript RT enzyme (Invitrogen) and RNase free water to make a final volume of 20 µl .The reaction mixtures were incubated for 60 minutes at 37°C in PCR machine. These reaction mixtures were then kept on ice immediately and stored at -20°C for long term storage.

PCR on cDNA

The cDNA so prepared from liver and udder tissues and the selected milk samples of Jersey Red Sindhi crossbred cows were used as templates for transcript expression profiling. Four separate reaction volume were prepared each using 2.5 µl PCR buffer, 50 mM $MgCl_2$,10mM dNTPs, 0.75 U *Taq* DNA polymerase, 0.4 mM

each forward and reverse primer (CathL1, CathL 2 CathL3 or CathL4, respectively), 2µl template cDNA and distilled water to make a final volume of 25µl. A pair of TbP primer was used as an internal control at final concentration of 0.4 µM/ µl in another PCR reaction to produce an amplicon. The PCR protocol was used as given by Whelehanet al.(2014). PCR for the amplification of TBP gene on cDNA was performed using PCR protocol given by Lisowski et al. (2008).

Electrophoresis

PCR products along with the 100 bp DNA ladder (Invitrogen) were electrophoresed in 1.2 % agarose gel intris borate EDTA buffer. A total of 5 µl of PCR product was mixed with 1 µl of 6(X) loading dye solution (Invitrogen). Tris borate EDTA buffer was used as medium in electrophoresis tank and electrophoresis was done at 75 V for 1 hour. The agarose gel was then stained for 30 minutes in water containing ethidium bromide (10 µl/ 100 ml water).The bands so obtained were visualized under UV in Gel Documentation System (Alpha Innotech, USA).

RESULTS

Somatic cell count in milk samples

Somatic cell count was performed for 40 milk samples of Jersey Red Sindhi crossbred cows.The milk samples with somatic cell counts of <0.4 million cells/ ml milk were classified as belonging to non-mastitic cows whereas the milk samples with high somatic cell counts *i.e.* between 10-50 million cells/ ml milk were classified to be from mastitic cows. Out of 40 milk samples, seven milk samples were selected for evaluating AMPs expression based primarily on varying somatic cell counts which were 1,35,975; 1,63,170; 21,48,405;42,69,615; 46,77,540; 48,40,710 and 49,22,295, respectively. The reason for selecting milk sample with increasing somatic cell count was to examine the role of neutrophills in production of various cathelicidins under the investigation.

Evaluation of primers on gDNA

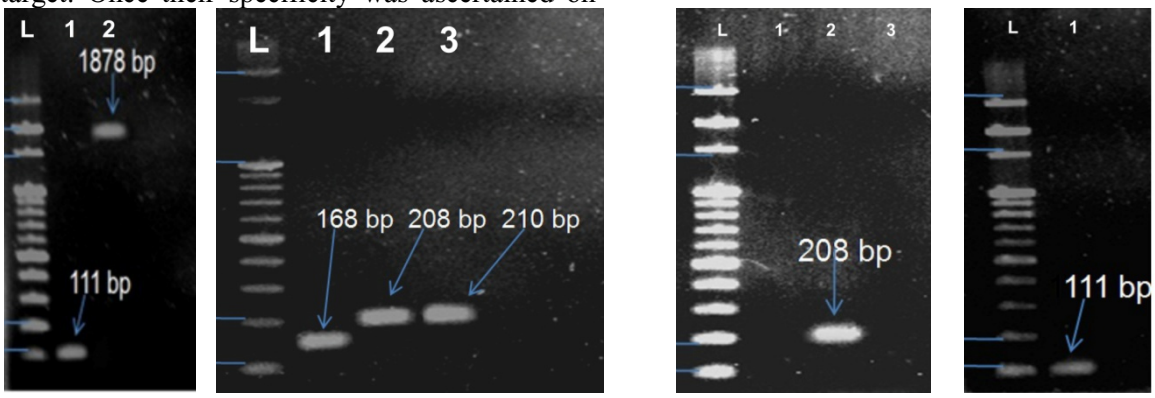
Correct sized amplicons from cathelicidin genes *viz:* cathelicidin1, cathelicidin2, cathelicidin3 and cathelicidin4, respectively, as mentioned in table 2 were obtained when gDNA isolated from liver tissue sample was amplified with pairs of CathL1, CathL2, CathL3 and CathL4 primers, respectively. Correct sized amplicon of TBP gene as shown in Table 2, (Fig.: 2(a)) was obtained when same gDNA isolated from liver tissue sample of Jersey Red Sindhi crossbred cow was amplified with a pair of TbP primer used as an internal control.

Table 2: Expected sizes of gDNA and cDNA amplicons of cathelicidin genes

Primer	gDNA amplicon's size (bp)	cDNA amplicon's size (bp)
CathL1 FP CathL2 RP	753	168
CathL2 FP CathL2 RP	797	208
CathL3 FP CathL3 RP	978	210
CathL4 FP CathL4 RP	900	184
TbP FP TbP RP	1878	111

The primers were tested with gDNA to see whether they are working and also to check whether amplicon sizes are different from that of amplicons of cDNA. All the primers worked very well on gDNA yielding larger amplicons as opposed to smaller expected amplicons from cDNA. This size difference allowed discrimination between gDNA amplicons and cDNA amplicons if cDNA would have been contaminated with gDNA. A pair of TbP primer was used as an internal control as TATA box binding protein gene is a general transcription factor present in all mammalian cells. This pair also produced amplicon of 1878 bp on gDNA as opposed to amplicon of 111 bp on cDNA (Fig. 2

a). So, all the primer pairs whether used for the amplification of cathelicidin genes or for the amplification of internal control house- keeping gene succeeded in amplification of their intended target. Once their specificity was ascertained on gDNA, then only these were used on cDNA prepared from total RNA isolated from liver and udder tissue and the selected milk samples of Jersey Red Sindhi crossbred cows.



a b c d

Fig 2: Expression of various cathelicidins in cow tissues

a : TBP gene amplicon on cDNA of liver tissue sample of Jersey Red Sindhi crossbred cow. Lanes L-100 bp ladder, 1-111bp, 2- 1878 bp TBP gene amplicon

b: Cathelicidin gene amplicon. Lanes L- 100 bp ladder, 1- 168 bp cathelicidin1, 2- 208 bp cathelicidin2, 3- 210 bp cathelicidin3

Fig 2: Expression of various cathelicidins in cow tissues

c: Cathelicidin gene amplicon. Lanes L- 100 bp ladder, 1- No cathelicidin1 amplicon, 2-208 bp cathelicidin2 amplicon, 3- No cathelicidin3 amplicon

d: TBP protein gene amplicon on cDNA of udder tissue sample of JerseyRed Sindhi crossbred cow Lanes L- 100 bp ladder, 1-111 bp TBP

Expression of AMPs in different samples

The expression of cathelicidin AMPs: cathelicidin1, cathelicidin 2, cathelicidin 3, and cathelicidin 4, respectively was studied in liver, udder tissue and the selected milk samples with varying somatic counts.

Correct size of amplicons were obtained for cathelicidin genes: cathelicidin1, cathelicidin2 and cathelicidin3, respectively as mentioned in Table 2, (Figure: 2(b) when cDNA of liver tissue sample was amplified with pairs of CathL1, CathL2 and CathL3 primers, respectively. Transcript of cathelicidin 4 gene was not obtained when cDNA of liver tissue sample was amplified with a pair of CathL4 primer. These results showed that cathelicidin 1,cathelicidin 2 and cathelicidin 3, respectively were being expressed in liver tissue sample of Jersey Red Sindhi crossbred cow but, cathelicidin 4 gene was not expressed in liver tissue sample of Jersey Red Sindhi crossbred cow used in this study. Correct sized amplicon was obtained for cathelicidin 2 gene as mentioned in table 1.2, (Fig. 2 (c)) when cDNA of udder tissue sample was amplified with a pair of CathL2 primer. This finding is an exception to the earlier findings of Tomasinsig et al. (2010) who did not obtain the expression of cathelicidin2 gene in normal udder tissue of cows. Cathelicidin1 and Cathelicidin 3 transcripts were not detected in udder tissue sample. These findings were in accordance with the earlier findings of Tomasinsig et al. (2010).Transcript of cathelicidin 4 as shown in table 1.2 was not obtained on cDNA of udder tissue sample of Jersey Red Sindhi crossbred cow. This finding is an exception to the earlier findings of Kosciuczuk et al. (2014), where the expression of cathelicidin 4 gene was

obtained in the udder tissue of cows. These results showed that cathelicidin 2 gene was expressed in udder tissue sample of Jersey Red Sindhi crossbred cow but cathelicidin 1,cathelicidin 3 and cathelicidin 4, respectively were not being expressed in udder tissue sample of Jersey Red Sindhi crossbred cow used in this study.

Correct size of TBP transcript used as positive control as shown in Table 2, (Fig. 2 (d)) was obtained on cDNA of udder tissue sample.

The cDNA obtained from the seven milk samples having somatic cell counts as 1,35,975; 1,63,170; 21,48,405; 42,69,615; 46,77,540; 48,40,710 and 49,22,295 respectively were amplified with pairs of CathL1, CathL2, CathL3 and CathL4 primers. Expression was not obtained with any of the CathL primer pairs. These findings were an exception to the earlier findings (Whelehan et al., 2014) where expression of cathelicidin1, cathelicidin 2, cathelicidin 3 and cathelicidin 4 was obtained in the milk of cows with varying somatic cell counts and Smolenski et al. (2014) obtained the expression of all bovine cathelicidin genes in milk of mastitic and non-mastitic cows.

Correct size of TBP transcripts were obtained when cDNA of selected milk samples were amplified with a pair of TbP primer in the internal controls in accordance with the earlier findings of Verbekeet al., (2015), who obtained the expression of TBP gene in milk of lactating cows.

DISCUSSION

Cathelicidins are host defense proteins involved in acute phase reactions. Their expression is indicative of occurrence of an infectious disease within a particular tissue or they may be normally present in these tissues. In bovine specie, milk component, udder and liver tissues besides other organs are important which affect the productive health of the animals. Therefore, these tissues were assessed under normal health status and at unhealthy status i.e with high somatic cell count in milk. It was concluded that variable expression of cathelicidin genes was present in Jersey Red Sindhi crossbred cows in liver and udder tissue. Expression of cathelicidin genes *viz* cathelicidin1, cathelicidin 2 and cathelicidin 3 was recorded in liver tissue of Jersey crossbred cow but expression of cathelicidin 4 gene was not detected. Expression of cathelicidin 2 gene was recorded in udder tissue but no expression of cathelicidin1, cathelicidin 3 and cathelicidin 4 genes was detected. In this study, the expression of cathelicidin AMPs could not be found in the selected milk samples, which was unexpected as many studies in the past have shown the expression of cathelicidins in Friesian-Jersey cross-bred cows milk (Smolenski et al., 2014) and Holstein-Friesian cows (Whelehan et al., 2014). The non-detection of AMPs in milk in this study could be attributed to difference in breeds, physiological status or low specific mRNA, However, the positive results for house-keeping gene were indicative that there was enough good quality cDNA recovery from milk samples thus ruling out any procedural faults with the protocol or any other quality issues with the reagents.

Conclusion

The study was conducted to determine the expression of cathelicidin genes *viz* cathelicidins1, cathelicidins 2, cathelicidin 3 and cathelicidins 4 inthe milk of Jersey Red Sindhi cross bred cows with varying somatic cell counts as well as udder and liver tissues. Expression of cathelicidins coding genes *viz*cathelicidins1, cathelicidins 2 and cathelicidins 3 was found in liver tissue but not of cathelicidins 4 gene. Expression of cathelicidins 2 gene was found in udder tissue but not of cathelicidins 1, cathelicidins 3 and cathelicidins 4 genes, respectively. Expression of cathelicidins1, cathelicidins 2, cathelicidins 3 and cathelicidins 4 genes, respectively was not found in the selected milk samples of Jersey Red Sindhi crossbred cows.

Acknowledgment

We acknowledge the help provided by the CSK HPKV, Palampur Livestock farm for allowing the sampling for the animals and the University for logistic support.

Conflict of interest

The authors do not have any conflict of interest.

Ethical approval

All procedures were followed for the ethical treatment of animals.

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