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Follicular Dynamics Theory in Programmed Breeding and Superstimulation of Cattle

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ABSTRACT

Programmed breeding has become an integral part of reproductive management in many dairy herds. A thorough understanding of oestrous cycle physiology and follicular wave dynamics in particular, is necessary for implementation of fixed time artificial insemination (FTAI). Individual variation within the animals in exhibiting follicular wave patterns poses a major problem in the outcome of oestrus synchronization nad superstimulation programmes. The follicular and luteal turnover in bovines during a cycle was highly unpredictable as it was governed by various factors. With the advent of real time ultrasonography it has become possible to visualize the various reproductive events, which provided a basis for synchronizing oestrus and ovulation with more precision. The outcome of prostaglandin based-, GnRH based- and progesterone based– oestrus synchronization protocols can be improved by positively manipulating the follicular dynamics. Better superovulatory response could be achieved by ensuring induction of follicular wave emergence and initiating FSH treatment on days before the deviation of dominant follicle of induced wave. It is suggested that the protocol of follicular wave synchronization could be successfully incorporated in the oestrus synchronization and superstimulation programme of cattle. The review deals with various recent protocols designed to control both luteal and follicular function that permit breeding with high pregnancy rates and superstimulatory treatment with improved response and embryo quality.

Keywords: Programmed breeding, oestrus synchronization, superstimulation, follicular dynamics, cattle

Fixed time artificial insemination (FTAI) has become an integral part of reproductive management in many dairy herds. FTAI protocols that synchronize follicle growth, corpus luteum (CL) regression and ovulation, result in improved reproductive performance, because all animals are inseminated whether they show oestrus or not. In general, these programs are designed to assist in obtaining pregnancies in groups of cows, primarily by restricting the intervals during which oestrus detection needs to be done, or by eliminating the need for oestrus detection. Although there are numerous controlled breeding protocols in beef and dairy herds, a thorough understanding of oestrous cycle physiology and follicular wave dynamics in particular, is necessary before one attempts manipulation of the oestrous cycle.

Basics of Follicular Dynamics

Various investigative techniques have been used to characterize the follicular development in farm animal species. The modern era of study of follicular dynamics by real time ultrasonography in cattle began with the classic publication by Pierson and Ginther (1984) and in the recent years our understanding of follicle growth and maturation has improved considerably since ovarian follicular development can be easily and accurately monitored on a day-to-day basis by ultrasonography *in vivo*. As the follicles develop towards the ovulatory stage, three features appear to be highly conserved across all species:

1) the sequence of events (recruitment, selection and dominance);
2) the sequential need for gonadotrophins (FSH

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for recruitment, LH for dominance) and in addition, specific follicles may also have variable gonadotrophin requirements (thresholds) and

3) the large variability of numerical parameters (number of waves per cycle, number of follicles per wave) as well as temporal requirements (time of selection, duration of dominance).

Follicular development in wave pattern

When patterns of follicle development at different physiological states are compared across species, follicular waves were detected in cattle, buffaloes, sheep and horses, suggesting that ovaries of all species operate on a wave basis unless they are prevented from doing so. The wave-like pattern of ovarian follicle development in cattle has been thoroughly described in *Bos taurus* (Ginther, 2000), *B.indicus* (Viana *et al*., 2000) and in *B.taurus* x *B.indicus* crossbred cattle (Satheshkumar *et al*., 2008a and 2008b). A brief summary of these studies showed that waves of follicular development occur at 7- to 8-day intervals throughout the oestrous cycle. Each wave produces a dominant follicle (DF) capable of ovulating if luteolysis occurs during its tenure of dominance. Thus a follicular wave comprises periods of emergence, growth, dominance and atresia or ovulation. The bovine oestrous cycle will normally have one or two non-ovulatory waves and an ovulatory wave.

Recruitment of follicular waves and selection of a dominant follicle is based on differential responsiveness to FSH and LH (Adams *et al*., 1992). Surges in plasma FSH elicit emergence of a follicular wave; FSH is subsequently suppressed by products of the growing follicles (i.e., oestradiol and inhibin). As a result, FSH is declining when growth profiles of the dominant and subordinate follicles begin to diverge (time of selection), approximately 3 days after wave emergence (Ginther *et al*., 1996). Within each wave, the follicle that becomes the DF is also the first to acquire LH receptors while subordinates which still require FSH to maintain growth undergo atresia. The DF grows for approximately 6 days and then enters a static phase. Suppression of LH as a consequence of progesterone secretion by the CL causes the DF to cease its metabolic functions after 2 or 3 days, and it begins to regress and FSH surges again. This FSH surge has no effect on the atretic DF, but is responsible for eliciting emergence of the next wave. The ovarian cycle then repeats itself. Luteal regression allows LH pulse frequency to increase; the DF increases its growth rate and produces higher concentrations of oestradiol which act in a positive feedback on the hypothalamo-pituitary axis, resulting in a surge of LH followed by ovulation.

There was a great variation in the proportion of animals exhibiting two- or three-wave cycles. Satheshkumar (2009) found that 67.77 per cent of crossbred cows exhibited varied follicular wave patterns between the subsequent cycles. Thus the occurrence of follicular wave patterns in bovines was not the same for each and every oestrous cycle of an individual animal and was highly unpredictable as it was governed by various factors like time of luteal regression (Ginther *et al.,* 1989), plane of nutrition (Murphy *et al.,* 1991), circulating FSH concentration (Adams *et al.,* 1992) and season (Badinga *et al*., 1994; Satheshkumar *et al*., 2015). These variations in follicular developmental patterns contribute for uncertainty or inconsistent response for the oestrus synchronization and superovulation protocols among the farm animal species. It is clear that follicle wave dynamics can have a profound effect on the efficacy of oestrus synchronization programs. Hence there is a need to optimize the timing of these protocols by positively manipulating the follicular and luteal dynamics.

Oestrus synchronization

The most common synchronization schemes in cows and buffaloes are limited to prostaglandin, GnRH and/or progestogen protocols. However, the variations with these approaches range from non-responsiveness to variability in time from treatment to oestrus and ovulation and / or ovulation of an aged oocyte. It is necessary to control follicular wave dynamics as well as luteal function in order to have a 'healthy' DF present at the end of treatment (Mihm *et al*., 1994). With the advent of real time ultrasonography it has become possible to visualize the various reproductive events over a prolonged period of time and without any interruption of normal physiological events, which will provide a basis for

synchronizing oestrus and ovulation with more precision.

Efficient oestrus control treatments should have the ability to effect

1) prevent the development of persistent DFs containing aged oocytes, and
2) recruit the future ovulatory follicle and synchronous ovulation of a growing DF.

The ***key is the induction of synchronized of follicle wave emergence***. So, oestrus synchronization treatment protocols need to induce atresia of all large follicles present on the ovaries whatever their developmental stage, resulting in recruitment of a new follicular wave, synchronous development of a new DF, and ovulation at a predictable time.

Ultrasound-guided follicle ablation is very efficacious in synchronizing follicle wave emergence but is difficult to apply in the field. Similarly, the induction of ovulation with GnRH or LH will effectively synchronize follicle wave emergence, but ovulation occurs in only 60 to 70% of cases. The administration of oestradiol benzoate in progestin-treated cattle effectively synchronizes follicle wave emergence for both superovulation and oestrus synchronization, but oestradiol benzoate is not available in many countries. The challenge now is to use knowledge of follicle wave dynamics to design alternatives.

Prostaglandin based protocols

Prostaglandin $F_2\pm$ (PG) has been the most commonly used treatment for synchronization of oestrus in cattle (Odde, 1990). However, induction of oestrus is distributed over a 6-day period (Macmillan and Henderson, 1983) after PG. In another study Bo *et al.* (2003) found that although 80–100 per cent of the cows treated with PG had luteal regression, only 60.0% were detected in oestrus and only 51.0% ovulated within 5 days of treatment. Wide variability in oestrus response and pregnancy rates after PG treatments is influenced not only by the responsiveness of the CL to the PG treatment (Momont and Seguin, 1984), but also to the stage of development of the DF at the time of PG treatment (Kastelic and Ginther, 1991). These results highlight the need for treatments that control luteal and follicular development to obtain high pregnancy rates to FTAI, avoiding oestrus detection.

Any DF has the capacity to ovulate provided the inhibitory effects of progesterone can be removed at an opportune time. Prostaglandins serve this function by destroying the corpus luteum (CL), however, PG has no direct effect on the normal pattern of follicular waves. It has been shown that, when progesterone concentrations in circulation begin to decrease, the DF that is in its growing phase goes onto ovulate, shortly afterwards (Lucy *et al.*, 1992).Thus, the stage of follicular development at the time of PG injection will affect the interval from injection to standing oestrus. Animals injected when the DF is in the growing phase will display oestrus within 2 to 3 days, whereas animals with aged or regressing DFs may

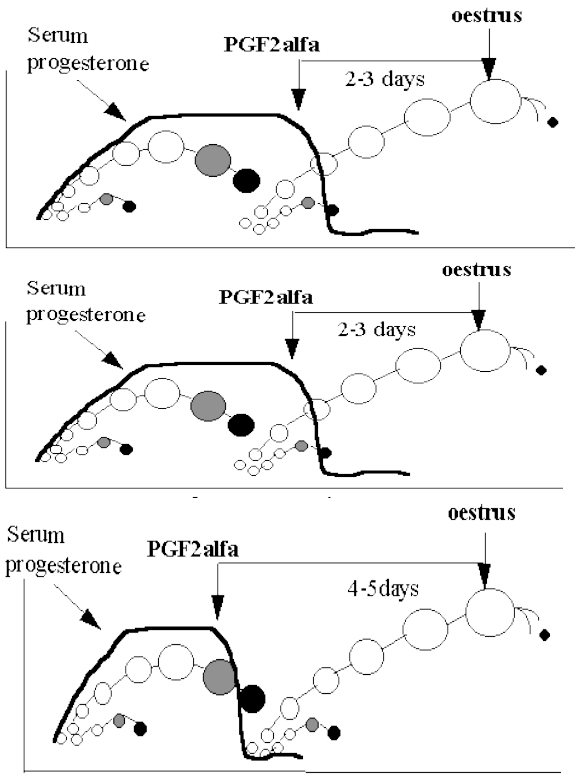

Fig.1: Variability in oestrus response after PG treatment

require 4 to 6 days before a new follicle can be recruited for ovulation (Fig.1). Thus, the interval from PG injection to oestrus and ovulation is highly variable due to differences between cows in the stage of follicular development at the time of PG injection. Synchronization was most precise

(75–80% in oestrus within a single 24 h period) when cows were injected in early dioestrus (7 days after oestrus; Day 7) or in late dioestrus (Days 15 and 16) and it was most variable among cows on Days 12 and 13 (Macmillan and Henderson, 1983).

Gonadotropin-releasing Hormone (GnRH)-Based Protocols

Prostaglandins (PG) alone do not provide acceptable synchrony, because the time to ovulation depends on the stage of development of the DF at the time of PG-induced luteolysis. Because of this, a treatment combining gonadotrophin releasing hormone (GnRH) and PG preparations is being introduced in the oestrus synchronization protocols (Ovsynch) for more precise results with greater degree of synchrony in oestrus and ovulation (Pursley *et al*., 1995; Barros *et al*., 2000). The initial GnRH injection expected to cause ovulation or luteinisation of the large follicle present at that time and synchronized the recruitment of a new follicular wave. At 7 days following GnRH injection, an injection of PG induced regression of the corpus luteum (CL) and allowed for final maturation of the synchronized DF. A second GnRH injection is given 36-48h after PG injection, synchronized the time of ovulation of the DF.

Ovulation failure in response to the initial GnRH injection in the Ovsynch protocol may result in reduced pregnancy rates following FTAI due to asynchronous ovulation (Vasconcelos *et al*., 1999). For example, premature oestrus between the first GnRH and the injection of PGF has been reported in 5.0 – 11.8% of cows (DeJarnette *et al*., 2004). In cows treated with the Ovsynch protocol 11.0 % ovulated before FTAI, 15.0 % did not respond to treatment with PGF, and 9.0 % did not ovulate after the second treatment with GnRH, indicating that synchronization rate was only 68.0 % (Colazo and Mapletoft, 2014). These variation in results obtained with Ovsynch was associated with the stages of the oestrous cycle and follicular status when treatment has been initiated (Thatcher *et al*., 2002).

The presence of a DF at the start of the Ovsynch protocol has been reported to be a strong positive indicator of the likelihood of conception in cattle.

i. Macmillan *et al.* (2003) stated that induction of ovulation using GnRH had been most successful only when injected after the deviation of the growing follicle and the establishment of dominance. By ultrasonographic study of follicular dynamics in Jersey crossbred cattle, Satheshkumar *et al*. (2012) concluded that irrespective of two- or three-wave cycles, the DF of the first follicular wave emerged between Days 0.8 -1.67, got deviated between Days 3.30 – 3.67 and reached its maximum size between Days 6.67 – 7.30 (Day0 – Oestrum).

ii. Moreover, LH receptor mRNA was expressed on granulosa cells when the DF was greater than 9mm in diameter and was first observed on Day 4 of the follicular wave, equivalent to Day 5 – 6 of the cycle (Xu *et al*., 1995) (Fig.2).

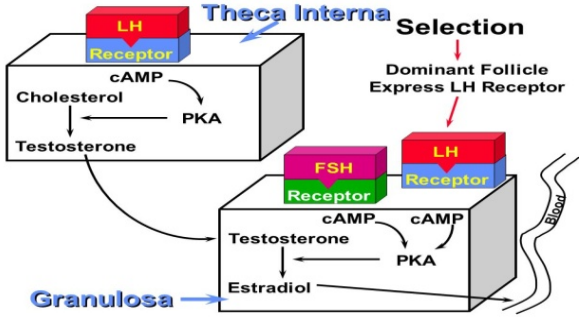

Fig.2: Expression of LH receptors in dominant follicle

Cattle in which GnRH was administered between Days 1 and 4 or Days 14 and 21 of the cycle had lower pregnancy rates than those treated at other times (Vasconcelos *et al.,* 1999). When GnRH is administered during metestrus (Days 1 to 3), the DF may not ovulate, and begins to undergo atresia at the approximate time that the PG is injected. The DF of the second wave (Days 13 to 17) may also not ovulate in response to the first GnRH treatment, and in the absence of ovulation, endogenous PG may cause luteolysis and ovulation before TAI, resulting in low pregnancy rates. From these studies, it was evident that the DF of the first wave established its dominance and was in growth phase between Days 4 and 6 of the oestrous cycle, a stage which was 100% responsive for GnRH resulting in emergence of new follicular wave by 48h**.** Similarly in Murrah graded buffaloes Dharani *et al*. (2010) found that

the application of Ovsynch protocol at growth phase of first wave DF (Day 6) exhibited better ovulation rate than that initiated at regression phase (Day 10) of the same (100% vs 33.33%).

Based on these factors various procedural alterations have been employed to improve the response to Ovsynch protocol.

"Presynch-Ovsynch": Presynchronization with PG is commonly used in dairy herds to ensure that cows are at the most appropriate stage of the oestrous cycle at the time of the first GnRH treatment. Pre-synchronization with 2 doses of PG, 14 days apart, and administration of the first GnRH 12 days after the second PG increase the probability that a LH-responsive follicle will be present at the time of the first GnRH.

"G6G": Bello *et al.* (2006) developed a novel presynchronization protocol that combines PGF and GnRH. The aim of this protocol was to increase the percentage of animals that respond to the first GnRH injection of the Ovsynch protocol by increasing the probability of an ovulatory-sized follicle at that time. Animals were treated with PG, followed 2 days later with GnRH administered at 6 days before the first GnRH in the Ovsynch protocol. When Ovsynch was initiated at 6 days after presynchronization with PG and GnRH, the percentage of animals that ovulated following the first GnRH of the Ovsynch protocol was 85% and pregnancy per AI was 50%.

"Double Ovsynch": Involves two Ovsynch protocols following one after the other, with the third GnRH treatment administered 7 days after the second. In the first study reported, Double Ovsynch resulted in a higher pregnancy rate than the Presynch-Ovsynch protocol in primiparous (65% *versus* 45%) cows (Souza *et al*., 2008). The improved pregnancy rate was probably due to the increased probability of a DF that would ovulate following the third GnRH, and the elevated circulating progesterone concentrations prior to the administration of PG.

Progesterone based protocols

Oestrus synchronization treatment protocols involving the use of progesterone / progestagens will only be successful if they prevent the development of a persistent DF, which might ovulate an aged oocyte. Reduced fertility following AI at synchronized oestrus could be the consequence of ovulation of an aged oocyte into a suboptimal oviductal environment (Revah and Butler, 1996) which has been shown to occur when progestagen treatments were initiated late in the oestrous cycle, when the CL had regressed spontaneously during treatment. Thus, the induction of a new follicular wave is important in progestin-based synchronization regimens, in order to prevent the development of a persistent follicle, which will result in reduced fertility following AI.

Bo *et al.* (1995) designed experiments to test the hypothesis that shorter acting oestrogen (oestradiol-17²), more effectively suppresses the DF when administered in combination with a progestogen ear implant than when given alone. In heifers treated with oestradiol-17² (5 mg) plus progestogen ear implants, they found that the DF ceased to grow 1 day after oestradiol-17² treatment and subsequently regressed, resulting in an early emergence of the next follicular wave (Day 5.2 $\pm$ 0.2). It was concluded that oestradiol-17² was more effective in inducing follicle suppression when combined with a progestogen ear implant.

Superstimulation programme

Gonadotropin induced superovulation is the basic and efficient method of obtaining multiple embryos from these genetically valuable females. However, the superovulatory response (SOR) is highly unpredictable and variable between treatments thus affecting the efficiency of the technology and limiting its practical application. Variability in ovarian response has been attributed to various exogenous factors such as donor parity and production status, season, hormone preparations and their dose. Above all, factors inherent to the donor animal i.e. the follicular status at the time of initiation of gonadotropin treatment influence the outcome of superovulation treatment. Success of a ovarian response is dependent on nature of dominant follicle (DF) and the availability of gonadotropin sensitive follicles at the time that treatment is initiated. Thus SOR was found to be higher when gonadotropin treatment was initiated at the time of follicular wave emergence (FWE). However ultrasonographic study of follicular dynamics

revealed that only 20 per cent of the estrous cycle (4 or 5 days) is available for initiating treatment at the time of wave emergence.

In conventional superovulation protocol the gonadotropin treatment was initiated in the mid luteal phase (Day 8 -12) of the cycle empirically, around the time of spontaneous emergence of second follicular wave (Wave II). However, there is great individual variation in the timing of the Wave II emergence in *Bos taurus x Bos indicus* crossbred cows and *B. taurus* cows that have two- or three-wave cycles. Thus, a wide variation in SOR observed with the conventional treatment could be attributed to cow-to-cow and cycle-to-cycle variations in follicular status at the onset of superstimulatory treatment.

Nasser *et al.* (1993) recommended first follicular wave (Wave I) as an option for initiating superovulation programme and stated that the time of emergence of Wave I (day of ovulation) was consistent when compared to subsequent follicular waves. Eventhough the follicles of the Wave I and II have the same capacity to respond to superovulation treatment, the follicles in the Wave I are exposed to an environment of lower progesterone (P_4) concentrations during the early luteal phase. So the follicles in the Wave I require additional exogenous supplementation to overcome the negative influence of lower systemic concentrations of P_4.

With the favorable hormonal milieu, follicles of Wave II could be targeted without any additional supplementation. However, difficulty in predicting the time of Wave II emergence poses a major problem. To obviate this problem, an alternative approach is to control the FWE and initiate the superstimulation treatment at the most favorable time that is optimal for recruited follicles to respond for exogenous gonadotropins (Fig.3).

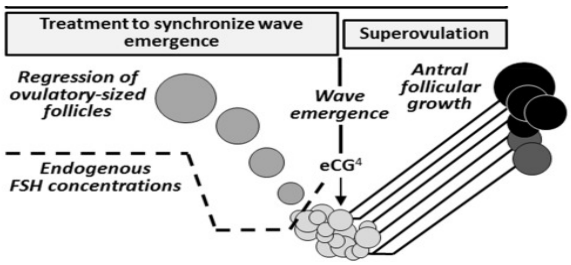

Fig.3: Synchronizing follicular wave emergence for initiating superstimulation treatment

The mechanical method (ultrasound guided follicular ablation) of inducing synchronized FWE when combined with superovulation protocol yielded better response, but the procedure warrants suitable equipments with skilled technician. Hormonal approach of estradiol (E_2) and gondotrophin releasing hormone (GnRH) agonist treatments have been widely used for self-appointed superstimulation protocols. When E_2 preparations were used, the time interval for emergence of a new follicular wave was found to be variable (3 – 6 days) and its availability is restricted in many countries. Moreover inclusion of controlled internal drug release (CIDR) devices in the superovulation protocols involves more animal handling and increases the cost of embryo production. On the other hand, with precise induction of FWE and easy application, GnRH agonists are promising alternatives for inclusion in superovulation programmes. However, previous reports have stated limited success in terms of SOR and embryo yield when GnRH pretreatment was included in superovulation schedule. Perusal of these reports revealed that GnRH was administered over a wide range of days randomly during a cycle which would not have necessarily induced ovulation of DF, a prerequisite for emergence of a new follicular wave. Further, Evans and Fortune (1997) stated that there was no difference between the dominant and subordinate follicles in the expression of mRNA for FSH receptors till second day after FWE. Considering these facts, it was hypothesized that better SOR could be achieved by ensuring induction of FWE and initiating FSH treatment on days before the deviation of DF of induced wave.

Satheshkumar *et al*. (2010) stated that administration of GnRH on Day 6 of the cycle and initiation of FSH treatment on Day 10 resulted in homogenous recruitment of healthy follicular inventories under a favorable endocrine environment leading to lesser degree of variation in SOR and embryo yield. In addition, with the recovery of increased percentage of transferable quality and better developed embryos than the conventional method, it is suggested that the present protocol of GnRH induced follicular wave synchronization could be successfully incorporated in the superstimulation programme of cattle.

Conclusion

Our expanding knowledge of ovarian function during the bovine oestrous cycle has given us new approaches for the precise synchronization and control of ovulation. Recent protocols, designed to control both luteal and follicular function, permit FTAI with high pregnancy rates, and the initiation of superstimulatory treatments at a self-appointed time, and provide opportunities to do FTAI in donors and fixed-time embryo transfer in recipients.

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All authors declare that there exist no commercial or financial relationships that could in any way lead to a potential 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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Neglected Causes of Canine Viral Enteritis: An Overview

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ABSTRACT

Acute viral gastroenteritis has attained the status of a global problem affecting both animals and humans. The situation is quite worse in the developing countries. With advancing knowledge several new viral agents having zoonotic-origin have been identified. The role of companion animals in transmitting pathogens to humans cannot be ignored. Like humans, the dogs are also prone to various infections. Although information is scanty, gastroenteritis is still considered to be a primary menace to dogs. Till now, more commonly rotavirus and coronavirus are being reported as the cause of diarrhea. In-depth molecular epidemiological studz`ies using state-of-art tools have shown emergence and spread of new viruses to larger geographical areas and in new animal host species. Nonetheless, viruses like astrovirus, picobirnavirus, kobuvirus, circovirus, etc being reported in dogs, their epidemiological profile, and impact remains under-estimated and underreported. Here, we have discussed the neglected gastroenteritis viruses of dogs which could pose a threat in the future as well as may lead to zoonotic threats to humans.

Keywords: Gastroenteritis, Virus, Canine, Zoonosis, Diagnosis

Introduction

The human population since the time of their initial settlement is dependent on companion animals and dogs remain the most trusted pets on the earth. Being companion animals, they come in close contact with humans and even share common space. The close interaction with humans sometimes helps in exchange of pathogens between the two host species (Summa et al., 2012). Numerous infectious and non-infectious causes are thus known to affect the health of dogs. Amid them, the gastrointestinal tract infections are of growing concern due to associated losses and disease burden in dogs. Exact estimates regarding losses due to gastrointestinal tract infections in dogs are missing (Malik and Matthijnssens, 2014). But, the number of viral agents associated with diarrheal disease has steadily increased since last few years and, still, the list of emerging viral pathogens is ever-changing.

Canine viral enteritis begins with setting up of vomition and diarrhea, specifically in puppies. It has been observed that several animals are affected simultaneously. The main diarrhea initiating factors in dogs include sudden dietary change, intake of contaminated food material, and certain microbial (bacterial, viral, parasitic or fungal) infections. Among these, viruses are recognized as important causes of enteritis. Definitive diagnosis requires laboratory confirmation, most often detection of viral particles in the stool. However, despite extensive diagnostic analysis, the cause of many diarrhea cases remains unresolved.

In virus-associated gastroenteritis in dogs, the focus of research remains restricted to rotavirus, coronavirus, and parvovirus mainly, while several other pathogens including picobirnavirus, calicivirus, kobuvirus, circovirus, bocavirus, etc remained less emphasized (Caddy, 2018). Of late, some new information has been accumulated relating to these viruses and their host species. Detection and characterization of newer enteric virus strains from different geographic areas are important for understanding the worldwide distribution, heterogeneity, and association of these viruses with enteric disease in dogs and other

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animal species. The availability of next-generation novel pathogens and documentation of etiologic agents of numerous infections, cracking the long-standing secrecies of many genetically diverse and rapidly evolving viruses. In the past few decades, molecular characterization of many of these gastroenteritis viruses has led to advances both in our understanding of the pathogens themselves as well as in the development of state-of-art diagnostics. Although the use of these highly accurate, reliable, and sensitive approaches to identify and characterize individual viral pathogens is in its infancy, it has lightened-up new avenues to review disease burden and contemplate new directions for adopting vaccination, improvements in public health measures and sanitary practices. Here, we have discussed the neglected gastroenteritis viruses of dogs which could pose a threat for zoonotic transfer.

i Canine Picobirnavirus infection

Picobirnavirus (PBVs), the exclusive member of the family *Picobirnaviridae*, since its first report in 1988 from humans (Pereira et al., 1988) has shown presence in several mammalian, avian and reptile species (Malik et al., 2014, Banyai et al., 2014, Gallagher et al., 2017). It is a non-enveloped, double-stranded, bi-segmented RNA virus. Segment 2 encodes the RNA dependent RNA polymerase (RdRp) and on the basis of sequence diversity in this gene, PBVs are further classified into 2 genogroups (GGs), GGI and GGII (Malik et al., 2014). Although, the pathogenicity of PBVs largely remains to be determined, studies in immune-compromised individuals suggest that PBVs are opportunist pathogens (Haga et al., 1999, Fregolente et al., 2009). They have been identified from fecal specimens owing to large amounts of virus occasionally shed through feces, and have also been detected in sewage samples (Hamza et al., 2011). As on date, the epidemiology of PBV is quite unclear, whether it is influenced by host-species restrictions or animals serves as a reservoir of infection for humans. The basis of initial PBV detection was RNA-electrophoresis but it was improved with the addition of nucleic acid based assay, in particular the RT-PCR proposed in the year 2000 (Rosen et al., 2000). As followed for several other viruses, a uniform nomenclature scheme is used for PBV, which denotes genogroups, host, country of origin, strain, year of isolation for a specific PBV identified and is written in this order. For example, GI/PBV/Dog/IND/NR-346/2017 specifies a PBV with genogroup I specificity, detected in canine species from India, with strain name NR-346, in the year 2017.

To date, there are only a few reports available describing detection and molecular characterization of PBVs from cats (*Felis catus*) and dogs (*Canis lupus familiaris*). The first evidence of a PBV in canine faeces was reported in 2001 (Volotão et al., 2001) from Brazil as a tri-segmented genome RNA virus. Subsequently, detection of double-stranded RNA viruses in fecal samples of dogs with gastroenteritis in Rio de Janeiro, Brazil was reported (Costa et al., 2004). Later PBV was reported in three dog samples positive by PAGE and two by RT-PCR and the sample sequenced from dog PBV strain (dog|BR-02|BRA|2004) was found closely related to the human genogroup I PBV1-CHN-97 (Fregolente et al., 2009).

The literature perusal confirms the scanty information on genomic organization of canine PBVs, though genome sequencing has been completed for a number of PBV isolates from humans, porcine, dromedaries and bovine (partial or complete) (Ganesh et al., 2014). During 2014–2015, a total of 42 fecal samples were collected from household dogs from the Caribbean island of St. Kitts (KNA). Complete gene segment-2 of one dog PBV strain from Caribbean island was sequenced (Navarro et al., 2017). The PBV isolate strain exhibited a high degree of genetic diversity with the only available partial gene segment-2 sequences (~200nt, ~65 aa) of the two dog PBV strains from Brazil (Fregolente et al., 2009). The complete gene segment-2 of canine PBV strain PBV/Dog/KNA/RVC7/2015 was 1689nt long, and exhibits a G+C content of 44.46%. The gene segment-2 retained the 5′(GUAAA) and 3′ (ACUGC) end sequences that are conserved in PBVs from other host species (Malik et al., 2014). Although the presence of PBV in dogs has further expanded our knowledge of PBV diversity, still there is need to explore the host specificity of the virus, cross-species transmission and host-pathogen interaction including their association

with respiratory infections as being reported in pigs (Smits et al., 2011).

ii. Canine Kobuvirus infection

Kobuvirus is a comparatively new designated genus of the family *Picornaviridae*. It includes three established species as Aichivirus A, Aichivirus B and Aichivirus C, along with 3 proposed species Aichivirus D, Aichivirus E and Aichivirus F (ICTV, 10th Report). In the species Aichivirus A, three kobuviruses namely, Canine kobuvirus 1, Feline kobuvirus 1and Murine kobuvirus 1 have been described (ICTV, 10th Report). The first kobuvirus in dogs was described in 2011 during a study of acute gastroenteritis in canine shelters in the United States (Li et al., 2011a) and subsequently, several reports from different countries like UK, Italy, Korea, China, Africa, Japan have reported (Melegari et al., 2018). Kobuviruses have also been detected in wild canine species like red foxes, golden jackal, side-striped jackal and spotted hyena (Olarte-Castillo et al., 2015; Di Martino et al., 2014) and wolves (Melegari et al., 2018), which can act as the reservoir. Reports of kobuvirus detection in various canine species of last five years suggest its emerging nature and need further attention. Besides its presence in gastro-intestinal tract, it has also been found in extra-intestinal tissues like brain, liver, lung kidney, etc in canine infections (Ribeiro et al., 2017). Recently, a multiplex detection method for canine distemper virus (CDV), canine parvovirus (CPV), and canine kobuvirus (CaKV) has been developed (Liu et al., 2018). But till date information are inconclusive to identify a clear role for canine kobuvirus 1 in relation to disease, suggesting that the pathogenicity might be low. These viruses have been involved in diarrheal disease but the indication for a primary pathogenic role remains contentious.

iii. Canine Bocavirus infection

Animal bocaviruses were first described in the early 1960s (Manteufel and Truyen, 2008). As on date, twenty one species of genus *Bocaparvovirus* are known and the canine minute virus (CMV) has been classified as one of the species carnivore bocaparvovirus 1 (ICTV 10th report).A novel protein, NP1, encoded by all the bocaviruses has no homologue in parvoviruses of other genera (Manteufel and Truyen, 2008). In CMV, theNP1 plays an important role at an early step in viral replication and is also required for the read-through of an internal polyadenylation site that is essential for expression of the capsid proteins (Fasina et al., 2015). These viruses generally infect the gastrointestinal tracts (Siegl, 1984). CMV was first isolated in 1967 and was associated with clinical disease in 1970, causing respiratory illness with breathing difficulty and enteritis with severe diarrhea, spontaneous abortion of fetuses, and death of newborn puppies (Binn et al., 1970; Carmichael and Binn 1981, Carmichael et al., 1994). Information is still limited on the carnivore bocaviruses.

iv. Canine Circovirus infection

Circoviruses are amongst the smallest circular single-stranded DNA genome viruses which are known to infect pigs and birds (Li et al., 2011b). Being hardy, they survive for a longtime in the environment. The canine circoviruses are more closely related to porcine circovirus than to the avian circovirus (Gillespie et al., 2009). The circovirus infections have been recognized in dogs with vomition and diarrhea, and were reported in 2012 for the first time while screening canine samples for newer mammalian viruses (Li et al., 2013; Decaro et al., 2014).The nucleic acid based tests on dogs with and without diarrhea has shown a prevalence rate up to 11%.The available reports confirm the seriousness of canine circovirus infection as sole or concomitant infection with other pathogens. The detection of this virus infection in healthy dogs points towards the evaluation of its role in disease.

v. Canine Calicivirus infection

Members of the family *Caliciviridae*, are important enteric and respiratory pathogen affecting both animals and humans. This virus family contains five genera, namely *Vesivirus* (VeV), *Lagovirus*, *Norovirus* (NoV), *Sapovirus* (SaV), and *Nebovirus* (ICTV, 10th Report). These are small, single-stranded, non-enveloped viruses of 7.4-8.3 kb RNA genome. Among these, NoV and SaV are well recognized for causing diarrhea both in human and animal species (Bank-Wolf et al., 2010). In contrast, very little is known about

calicivirus infection in dogs. In 1985, a calicivirus was isolated from the faeces of a dog with diarrhea in the United States (Schaffer et al., 1985). Later, it was specifically classified into VeV genus after sequence analysis (Roerink et al., 1999). Among different caliciviruses, VeV is widely prevalent in dogs in Japan and Korea, though have not yet been established as an enteric pathogen for dogs (Soma et al., 2015). The first canine norovirus (CaNoV) was identified in 2007 in Italy, from a young dog with a 4-day history of gastroenteritis (Martella et al., 2008). Among six established groups of Norovirus (GI-VI), GIV and GVI NoVs have been detected in dogs, which on phylogenetic analysis appear to be grouping with lion and human noroviruses (Martella et al., 2008). Canine sapovirus (CaSaV) was first identified from canine diarrhea samples in 2011 by next generation sequencing (Li et al., 2011a). As yet, no epidemiological information is available for calicivirus infection in dogs but interspecies transmission reports are available from decades ago (Humphrey et al., 1984). The major HuNoV genogroup GII was also detected in the feces of three dogs, which suggests the role of canine species affecting public health and probable zoonotic infections (Summa et al., 2012).

vi. Canine Astrovirus infection

Astroviruses (AstV) are small star-shaped viruses and belong to the family *Astroviridae,* contain positive sense single strand genome of 6.4-7.3 Kb size. The family comprised of two genus namely *Avastrovirus* (avian species) and *Mamastrovirus* (mammalian species). AstV was first detected in canine species in the year 1980 in beagle pups with diarrhea in the United States in mixed infection with canine parvovirus type-2 (CPV2) and canine coronaviruses (CCoV) (Williams et al., 1980). After its first detection, the detailed molecular characterization was reported in 2009 (Toffan et al., 2009). Full-genome published in 2015 confirms its 6.6 Kb genome and three open reading frames. Canine AstVs appear to be widespread geographically and have been identified in the faeces of dogs with and without diarrhea (Caddy, 2018). They occur either alone or in co-infection with other enteric viruses. Their prevalence is considerably more in pups with gastro-enteric disease than in asymptomatic animals (Martella et al., 2011). Recently, three lineages in canine AstVs have been described by Zhou et al (2017). There are several molecular detection methods available for the specific canine AstV detection (Martella et al., 2011). Detection of novel AstV from human suggests presence of its animal reservoir (Banyai et al., 2010). So, there is an urgent need of large-scale molecular detection and genetic analysis of both human and animal AstVs. The shedding of virus has been substantiated to correlate with gastroenteric signs in naturally infected dogs. Astroviruses have been implicated in enteritis in several animal hosts but their pathogenicity in the dogs is still uncertain, for which additional animal experiments are obligatory to better comprehend the pathogenic role of astroviruses in dogs

Conclusions and future prospects

Identification of the novel viral agents has been driving the generation of methods to provide a better healthcare, and the diagnostic methods are evolving with the growth of the industry. Overall, there is an urgent need to summarize the current knowledge on neglected enteric viruses including taxonomy, biology and viral characteristics, and other aspects of infection including epidemiology, clinical picture, host species diversity and laboratory diagnosis. The possible transmissibility of some viruses between dogs and humans should also be assessed. On priority, the zoonotic consequences of these viruses detected in patients with gastroenteritis that resemble and cross-react with enteric viruses need to be clarified. The importance of continual vigilance for neglected but potential enteric viruses warrants to be highlighted so as to alert the researchers to explore the existence of these viruses in animals and their contribution to the diarrheal disease complex. The availability of such type of information is must for the adoption of appropriate and suitable control measures. The evolution of newer enteric viruses has opened up new avenues to reassess the disease burden, examine their molecular epidemiology, and consider new directions for their prevention and control through vaccination. The recent introduction of a rotavirus vaccine has considerably reduced the number of infected human infant, and thus has seta milestone for the future development for a safe and highly effective

vaccine against canine enteric infectious agents which could prevent cases of severe diarrhea and reduce mortality from this disease.

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Ethical approval

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

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Therapeutic Application of Lytic Bacteriophages in Mastitis Caused by *Pseudomonas Aeruginosa*

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ABSTRACT

Mastitis by *Pseudomonas aeruginosa* is opportunistic and environmental in nature. The treatment often fails due to higher antibiotic resistance and many a times end with recurrence or chemical treatment of affected quarter. Hence lytic bacteriophages were studied as an alternative therapeutic approach to antibiotics. For this purpose lytic bacteriophages were isolated using double agar overlay method using prophage free culture of *Pseudomonas aeruginosa*. Five lytic phages showing collective 100% host range were further characterized biophysically. The phages resisted pH 4, 7, 9 and temperatures of 16°C, 37°C and 42°C . On *in vivo* therapeutic study in mice, the lytic phage mixture at MOI 10^1 has decreased the inflammation of mammary gland effectively. The study revealed the application of lytic bacteriophages is worth as therapeutic agent and need further evaluation.

Key words: *Pseudomonas aeruginosa,* mastitis, lytic bacteriophages, therapy, mice

Introduction

Pseudomonas aeruginosa is one of the causative agents frequently isolated from bovine mastitis in buffaloes. Environmental mastitis by this organism increases somatic cell counts and decreases other milk components such as milk fat. This leads to the economic impact on the farmer (Park *et al*., 2007). The prevalence of *Pseudomonas aeruginosa* in subclinical mastitis is 3-5.4% (Benerjee *et al*., 2017, Kavitha *et al*., 2016). Furthermore, the organism uses a wide range of virulence factors like type III secretion system, which effectively damages tissues (Lyczak *et al*., 2000). In addition, the organism has the ability to survive in most environments (Erksine *et al*., 1987) and also exhibits intrinsic multidrug resistance (Melchior *et al*., 2006). The cure rate of mastitis caused by Pseudomonas is zero (Wagner and Erksine, 2009) with antibiotics. In this perspective an alternative therapeutic approach was studied using lytic bacteriophages in experimental mice model.

MATERIALS AND METHODS

Bacterial strains and prophages

The *Pseudomonas aeruginosa* (ATCC ® 27853™) was used as a standard culture along with the isolated *Pseudomonas aeruginosa* from the clinical cases of mastitis, for the purpose of the isolation of the lytic bacteriophages. In order to use these organisms as host, presence of prophages was observed using the DNA damaging antimicrobial agent mitomycin-C as described by Miller (1998).

Bacteriophage isolation

For isolation of bacteriophages by large scale screening, sewage samples were obtained from the places in and around buffalo farms where there was a possibility of obtaining sewage having more organic matter. While collecting 25 samples, the samples were collected from places where there is lesser chances for disinfectants or other chemical contamination. The collected sewage samples were centrifuged at 10,000 rpm for 10 min and then the supernatant was filtered using 0.45 µm filter. To this equal volume of 100 mM Sodium chloride and 8 mM Magnesium sulphate and 1M Tris HCl pH7.5 (SM buffer) and *Pseudomonas aeuroginosa* ($1.5x10^8$CFU/ ml) were added and incubated in orbital shaker incubator at 37°C for 24 h. After incubation, the suspension was centrifuged at 10,000 rpm for 10 min and filtered through 0.45µm filter. This filtrate was used to estimate the phage population by using double

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agar overlay method using bottom nutrient agar (with 2% agar). Then the top agar was prepared using 0.5 ml of the filtrate, one millilitre of the host culture ($0.5x10^8$ cfu/ml) and 1.5 ml of SM buffer and incubated at 37°C for 20 min. To this suspension, 3 ml of nutrient agar (2%) was added to make final concentration of 1%agar and layered on bottom agar. After solidification, the plates were incubated at 37°C for 24hrs and observed for the formation of clear plaques.

Purification of bacteriophages and microbiological characterization

From the pool of bacteriophages that were obtained on primary isolation, single plaques revealing clear plaque morphology and wide lytic zone were obtained using sterile toothpick, then inoculated into 2 ml of nutrient broth having $0.5x10^8$ cfu/ml of host culture and incubated at 37°C for 24 h in orbital shaker incubator. Later it was centrifuged and the supernatant was subjected to double agar overlay as described and the same was repeated thrice sequentially in order to obtain single lytic bacteriophage.

Among the isolated bacteriophages, the host range of five lytic phages was observed using spot assay as described by Santos *et al*. (2011). Then these bacteriophages were multiplied further and stocks were prepared.

Biophysical characterization

The obtained bacteriophages at multiplicity of infection (MOI) one by taking equal concentration of the bacteriophage and bacteria and were subjected to temperatures 16°C, 37°C, 42°C and pH 4, 7, 9 for a period of 4 hours by changing the temperature of incubation and pH of SM buffer, respectively and the decrease in bacteriophages count was observed using double agar overlay method at time intervals of 30min, 1, 2, 3 and 4hrs.

***In vivo* lytic activity of bacteriophages**

Female Swiss albino mice, aged 40 days and under lactation, were selected and grouped into control, infected and treatment groups. Each group had six mice, the infected group received 100 µl of $3x10^8$ cfu/ml of organisms whereas the treatment group received 100 µl of both the organism ($3x10^8$ cfu/ml) and endotoxin free (Proteospin endotoxin removal kit, Norgenebiotek) lytic phage cocktail ($3x10^9$ pfu/ml) by intramammary route. During the experiment the body weights of the mice were recorded and milk was also collected from three groups of mice. The therapeutic effect of the bacteriophages was estimated by conducting histopathology of the mammary gland as well as the total microbial count of milk using standard plate count method.

RESULTS

Bacterial strains and prophages

A total of eight *Pseudomonas aeruginosa* isolates (including reference strain) were used for lytic bacteriophages isolation and characterization. Exclusion of temperate phages was done using Mitomycin C induction of prophages.

Isolation of lytic bacteriophages

On an average 50 to 100 bacteriophage plaques were obtained on initial isolation from each sewage sample. Among these five lytic bacteriophage plaques showing clear plaque morphology of more than 3 mm were selected (Fig. 1). The selected phages ϕPA1, ϕPA2, ϕPA3, ϕPA4 and ϕPA5 were purified and stock cultures were prepared.

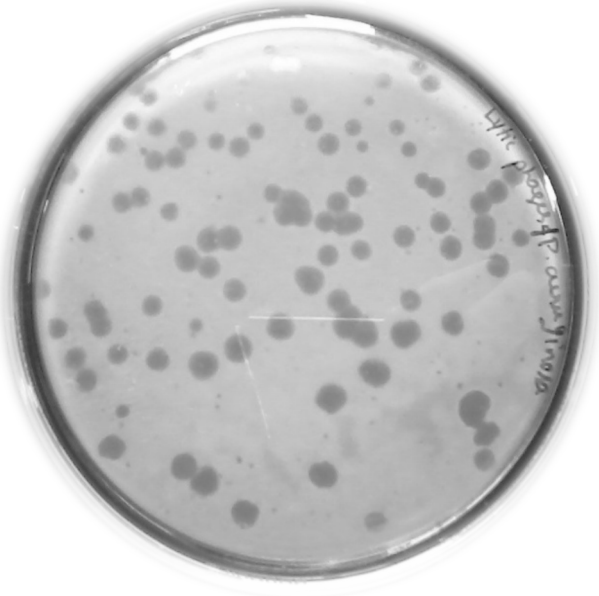

Fig. 1: Isolation of lytic bactieriophages

Microbiological characterization

The isolated phage host range was ascertained at multiplicity of infection 1. The phages revealed a host range of 92.5 to 93.75% with a collective host range 100% (Fig. 2).

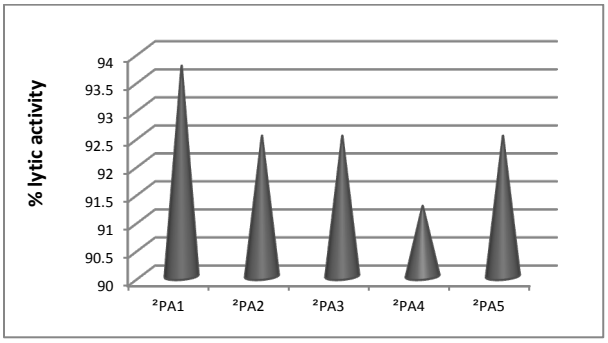

Fig. 2: Host range of lytic bacteriophages-percentage lytic activity

Biophysical Characterization

The lytic bacteriophages exerted stability at pH 4, 7, 9 and temperatures at 16°C, 37°C and 42°C. At multiplicity of infection 1 the phages were stable and exerted complete lysis. It was observed that there is increase in titre at pH 7 and temperature 37°C in comparison to acidic, alkaline and altered temperatures. Further it was also observed that among the lytic bacteriophages the ϕPA1 exhibited good stability by increasing its titre at the different pH and temperature (Fig. 3).

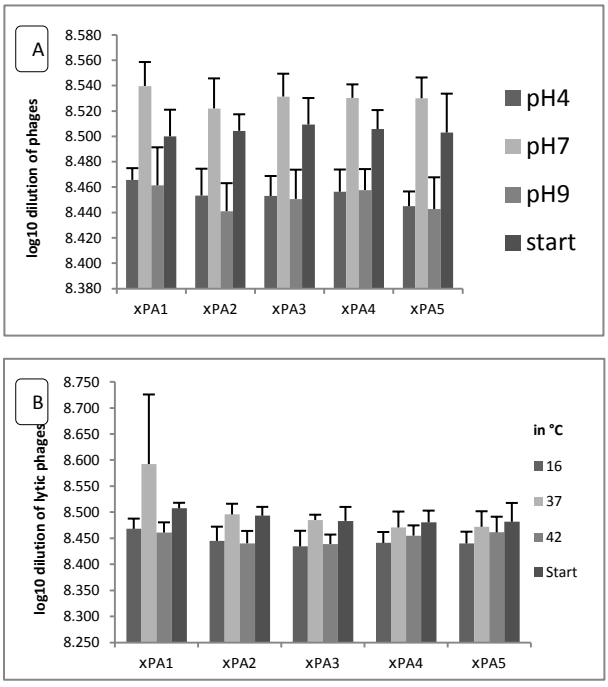

Fig. 3: pH (A) and thermo-stability (B) of lytic bacteriophages

In vivo lytic activity of the bacteriophages

Lactating Swiss albino mice were injected with bacterial culture and treatment group received bacteria along with bacteriophage cocktail (ϕPA1, ϕPA2, ϕPA3, ϕPA4 and ϕPA5). The infected mice revealed inflammation of mammary gland with swelling and redness. In comparison to the infected group, the treatment group appeared clinically normal. Control mice were normal and healthy. When the milk from both infected and treatment group was subjected for total bacterial count there was a decrease in the number of organisms in the treatment group as compared to the infected group. The tests of significance (Student t test) indicated significant difference between infected and treatment group at 24 h and 48 h ($P<0.01$). At 72 h of post-treatment, there was complete decline in the total bacterial count in bacteriophage treated group. It was also evident from histopathology that the neutrophil infiltration was also decreased after three days of post treatment (Fig. 4; Table 1).

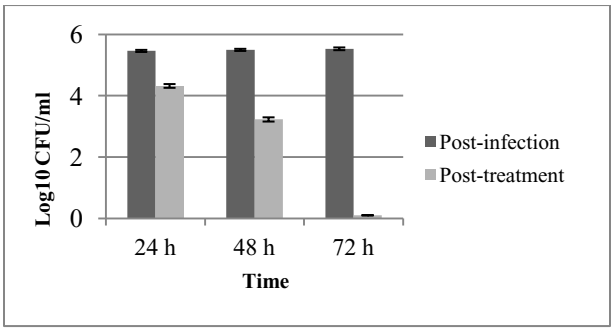

Fig 4: Total microbial count of milk in infected and treated group

Table 1: The results of the *in vivo* lytic activity of the bacteriophage mixture in mice

Conditions	Control group	Infected group after 72h	Treatment group		
			24h	48h	72h
Swelling and discoloration of mammary gland	-	+++	++	+	-
Dull and depression	-	+++	++	-	-
Average bacterial count as cfu/ml	-	$3.4x10^5$	$2.1x10^4$	$1.7x10^3$	-
Histopathological changes	-	+++	After 72 h reduced neutrophils		

Discussion

The emergence of antibiotic resistance in pathogenic organisms is becoming crucial which in turn prioritizing alternative anti-infection modalities. One such alternative is the application of lytic bacteriophages. In order to isolate lytic bacteriophages as therapeutic agents; it necessitates the use of host bacteria. The source of the bacteria was logically determined based on the target of phage therapy *i.e.* Pseudomonas mastitis in buffaloes. A total of seven *Pseudomonas aeuroginosa* isolates with one ATCC culture was narrowed down for the purpose of the isolation and characterization of the lytic bacteriophages. The host bacteria under Mitomycin C excluded the prophages. The exclusion of prophages is essential to avoid transfer of virulence as well as antibiotic resistance genes (Merabishivili *et al*., 2009).

Once suitable host bacterium was selected lytic bacteriophages were isolated from sewage influent as it yields a wide diversity of phages (Synnott *et al*., 2009). An average of 50-100 lytic bacteriophage plaques were obtained from each source. However five lytic bacteriophages (ϕPA1, ϕPA2, ϕPA3, ϕPA4 and ϕPA5) were selected. The plaque morphology was studied using same medium and conditions. The phages showing a diameter of 3 mm to 5 mm were selected. The phages with plaque morphology of 3 mm and above belong to the family Podoviridae (Kesik-Szeloch *et al*., 2013). Further the isolated phages tested on the lawn of different isolates of *Pseudomonas aeruginosa* in order to assess host range. The host range of five lytic bacteriophages ranged between 92.5 to 93.75%. The collective host range is 100%. In order to overcome the host specificity of lytic bacteriophages, the collective use of phages as cocktail was also suggested by many workers (Smith and Huggins, 1983; Smith *et al.,* 1987; Atterburry *et al*., 2007; Merabishvili *et al*., 2009).

Further, the phages on biophysical characterization revealed stability through acidic, alkaline pH as well as temperature variations. Alsaffar and Jarallah (2016) also observed stability of *Pseudomonas aeruginosa* phages at different pH and temperatures. However there is decrease in log concentrations of the phages at pH 4,9 and temperature 20°C and 42°C. But, this decrease didn't affect the lytic activity of phage through different pH and temperatures. Similar results were observed by Santos *et al*. (2011). Moreover, the increase in the phage concentration over initial concentration may be due to the effective multiplication of the host bacterium at pH7 and temperature 37°C which was in concurrence with OFlynn *et al*. (2004).

Prior to the application of phage cocktail as therapeutic agent, endotoxins has to be removed from the phage mixtures which are the common components that were release into the solution because of bacterial lysis due to phage lytic activity. As the endotoxins were proinflammatory so the endotoxin removal in bacteriophage cocktail was also supported by Merabishvili *et al*. (2009).

The phage mixture at multiplicity of infection 10^1 was used for the therapeutic application. Under *in vivo* studies in comparison to the infected group,

the treatment group revealed reduction in swelling and discoloration of mammary gland, improved health condition, reduced pain. In addition to the improvement in clinical condition the total bacterial count of milk prior to treatment and after treatment was also reduced. Moreover, the infiltration of neutrophils was also reduced in treatment group due to reduction in inflammatory process. Similar experiments in the application of lytic bacteriophages was also applied in the treatment of *Pseudomonas aeruginosa* in burn wounds cases by McVay *et al*. (2007), cystic fibrosis by Morello *et al*. (2011). Chilamban *et al*. (2004) observed recovery of animals from induced mastitis after 72hrs of intramammary inoculation. The animals revealed complete reduction in swelling of mammary gland and return to normalcy by gross and histopathological observations. Similar to the experiments discussed treatment of mastitis by *Staphylococcus aureus* in mice models using lytic bacteriophages, was also studied by chilamban *et al*. (2004).

In conclusion, the study demonstrated that lytic bacteriophages can be used significantly to reduce mastitis by *Pseudomonas aeruginosa* in mice models. The results of the study were promising, although further work needs to be undertaken to determine real life setting. Further it also needs the study of Endolysins as therapeutic agents to overcome host specificity of the lytic bacteriophages.

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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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Differential Expression of DRB1 Gene Transcripts in Harnali Sheep Exposed to *Haemonchus Contortus*

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ABSTRACT

Gastrointestinal nematodes constitute a major cause of morbidity and mortality in grazing ruminants. The Major Histocompatibility Class II complex plays an extremely important role in the resistance and susceptibility of sheep against *Haemonchus contortus* . In this study the differential expression of DRB1 gene transcripts in low and high fecal egg count animalswere assessed by using SYBR-Green real-time PCR. Animals were selected on the basis of fecal egg count (FEC) of *H.contortus*. Blood was collected from jugular vein and mRNA was extracted by trizol method. Further, RT-qPCR was done to analyze the differential expression of DRB 1 exon 2 in Low FEC and high FEC animals. The expression level of DRB1 gene transcript was found to be higher in low FEC animals as compared to the high FEC animals.

Key words: *Haemonchus contortus*, faecal egg count, DRB1 gene, real-time PCR, mRNA expression.

Introduction

Gastrointestinal nematode (GIN) infection restrains the growth of small ruminant industry worldwide. *Haemonchus contortus* is a blood sucking nematode that causes severe blood loss which can lead to anaemia, anorexia, depression, loss of condition, and eventually the death. *H.contortus* is of high economic significance due to its high prevalence and blood sucking habit (Notter *et al*., 2003), especially under warm and wet conditions. Major Histocompatibility class (MHC) is one of the candidate and several studies have confirmed the association between MHC alleles and nematode resistance (Sayers *et al.*,2005;Valilou *et al.*,2015); resistance to *Cystic echinococcosis* (Shen *et al* ,2014; Li *et al.,*2010); and footrot resistance (Valilou *et al.*, 2016) in sheep and goats. Besides this, MHC region also associated with economically important traits such as growth and ewe lifetime prolificacy (Cinar *et al.*, 2016).

The most polymorphic locus among MHC class IIa gene is DRB1. MHC class II molecules are found on the surface of antigen-presenting cells (APC), mainly on B lymphocytes, macrophages and dendritic cells). They are responsible for presenting antigens (extracellular proteins and parasites) to CD^{4+} T lymphocytes. Several genetic markers of disease resistance have been identified, including an allele (DRB1*1101) of the MHC-DRB1 locus. Sheep carrying the DRB1*1101 in the second exon has shown resistance to the GIN *Teladorsagia circumcincta* (Hassan *et al*., 2011). Studies on the evaluation of the expression of DRB1exon 2 in sheep infected with *H.contortus* are very few. Therefore, the objective of this study was to quantify the expression of *DRB1* (Exon 2) in low and high FEC of *H.contortus* in Harnali sheep.

MATERIALS AND METHODS

Blood samples

Blood samples were collected from six month old Harnali (cross of Nali, Corridale and Russian Merino) lambs maintained at sheep breeding farm of Department of Animal Genetics and Breeding, College of Veterinary Sciences, LUVAS, Hisar after taking permission from IAEC (Agenda item No. 16/16/1/13). Blood samples (1.5 ml) were collected in centrifuge tube containing 0.1 ml of 0.5M EDTA as anti-coagulant from eight lambs including, four animals each of high fecal egg count(more than>2000 EPG) and low fecal egg count(less than <500 EPG) and. One ml of blood was taken in 2 ml micro-centrifuge tube, centrifuged at 3000 rpm for 30 minutes and then plasma was removed by pipetting leaving the pellet behind. The pellet was mixed with 2

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volumes of RBC lysis buffer and kept for 20 minutes at 37°C followed by spinning at 2000 rpm for 20 minutes at room temperature. Supernatant was carefully discarded and ultimately WBC pellet was obtained and immediately used for total RNA extraction.

Extraction of RNA and synthesis of cDNA

Total RNA was isolated from the blood by using TRIzol® (Ambion) reagent as per the manufacturer's instruction with minor modifications. Total RNA was quantified by Qubit® 2.0 fluorometer (Invitrogen).Reverse-transcription was carried out from the total RNA isolated using TRIzol reagent with random decamer supplied along with cell to cDNA kit (Ambion).The reaction mixture of 20µl included 1µl of dNTPs (10mM each), 13µl of RNA, 1µl of Random decamers (Ambion), 0.5µlof Ribolock (Fermentas),0.5µlof M-MuLV-RT (200 IU/µl) (Promega)and 4µl of5X RT buffer (Promega). In a sterile RNase free microcentrifuge tube total RNA and random decamers were mixed and heated at 85°C for 3 minutes to melt the secondary structures within the template. Then rest of the components of reaction mixture was added in the tube. Reverse-transcription was carried out at 25°C for 10 minutes, 42 °C for 60 minutes in a programmable thermal cycler. The reverse transcriptase was heat inactivated at 72 °C for 5minutes. The cDNA was stored at -20 °C till further use.

Primers used in the study

The 110 bp fragment of the DRB1 gene was amplified using primer pair (Table 1) which was self-designed usingPrimer BLAST software from NCBI. The 91 bp fragment of the GAPDH gene was amplified using primer pair designed by using sequence of cattle NM_001034034.2 (Table 1).

Table 1: Sequence of the primers used in real-time PCR experiments

Gene	Primers	Sequence (5-3')	Product size
DRB 1 gene (Exon 2)	Forward Reverse	CAAGTACTGGAACAGCCAGAAGGA CTCGCCGCTGCACAGTGAAAC	110bp
GAPDH gene	Forward Reverse	GCAACAGGGTGGTGGACC ACTCTTCCTCTCGTGCTCC	91bp

Real-time PCR

The temporal expression level of DRB1 gene was analyzed by real-time PCR protocol using 7500 standard real-time PCR (ABI, USA). The reaction for the target DRB1 gene and the endogenous control GAPDH gene was carried out in triplicate for each sample including negative controls i.e non template control (NTC). Real-time PCR was performed using EvaGreen qPCR Mastermix-low ROX from G-bioscience. The reaction was carried out in a 20µl reaction volume using 10µl of EvaGreen qPCR mastermix-low ROX, 0.4µl of each primer (10 µM), 7.2µl of nuclease free water and 2.0µl of template. The cyclic conditions of qPCR were of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec, and 60°C for 60 sec with dissociation step at 95 °C for 15 sec, 60 °C for 60 sec, 95 °C for 15 sec and 60 °C for 15 sec.

Fold change in expression

Fold change in expression of DRB1 gene in low FEC and high FEC animal group was studied using Livak's method of relative quantification i.e "" C_T Method (Livak and Schmittgen, 2001). *GAPDH*, a house keeping gene was selected as a endogenous control. The average C_T (Threshold cycle) value obtained for the *DRB1* (target) gene was normalized to *GAPDH*.

Results and discussion

DRB1 and *GAPDH* genes were successfully amplified using designed set of primer (Fig.1a).The single peak of dissociation curve (Fig.1b & c) for amplicons of both *DRB1* and *GAPDH* genes revealed desirable amplification which was further confirmed using agarose gel electrophoresis. The fold change in expression of *DRB1* gene transcripts in low and high FEC animals was obtained after two tier normalization of average C_T value of *DRB1* (target) gene with *GAPDH* (endogenous control) and consideration of high FEC animals group as calibrator sample. The data obtained is described in table 2 and Fig.1d.

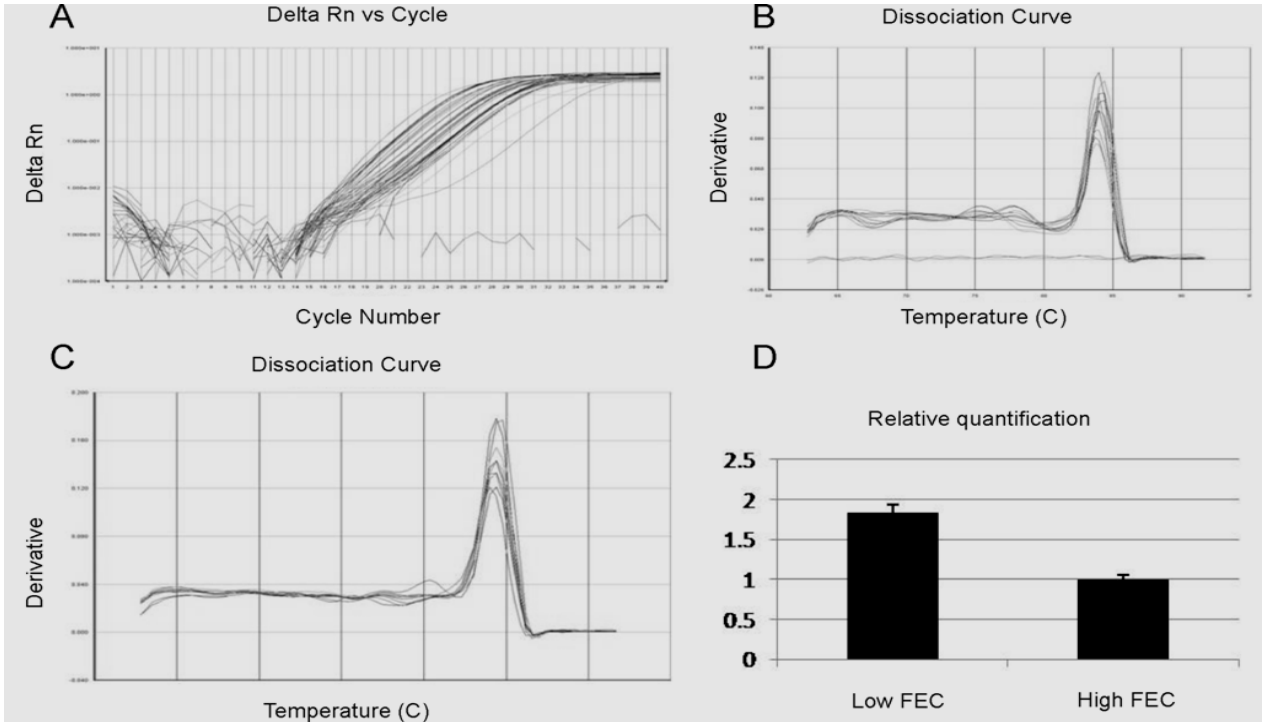

Fig. 1 Real-time PCR of DRB1 gene. Amplification curve of DR1 and GAPDH (a); Dissociation curve of GAPDH gene (b) and DRB1 gene (c); Relative expression o DRB1 transcript in low FEC and high FEC animals in sheep.

Table 2: Relative quantification of DRB1 gene transcript in low and high FEC animals

Animal groups	Average ” C_T (DRB1)	”” C_T	Relative quantification (RQ) $2^{\text{””Ct}}$
Low FEC	-1.019	-0.881	1.841
High FEC	-0.138	0.000	1.000

The relative abundance of DRB1 mRNA in blood was found to affect the FEC of *H.contortus in sheep*. The expression level of DRB1 mRNA was found to be higher (1.8 fold) in low FEC animals as compare to high FEC count animals (Fig.1d). Keane *et al*. (2007) analysed transcriptome of duodenum tissue of nematode resistant and susceptible Perendale sheep. They analysed almost 300 genes that were differentially expressed in both the groups. Among MHC genes, three genes were highly expressed in resistant animals i.e., *Ovar-DQA1* (8.4fold), *Ovar-DQB1 (*2.7fold) and *Ovar-DRA* (1.3fold).. Through this study they also exhibit the advantages of expression study in polymorphic variants of quantitative characters. .In another study by Corley &Savage, 2015 found

that in Boer and Spanish goats breed naturally resistant animals (> 18 PCV; >2000 FEC) to *Haemonchus contortus* had elevated expression of the DRB1*1101 than the susceptible (< 18 PCV; > 2000 FEC), whereas Myotonic goats naturally susceptible to *Haemonchus contortus* showed elevated expression of the DRB1*1101 allele. Through these studies it can be understood that there is a correlation between DRB1 locus expression with *H.contortus infection. H.contortus* continues to be a problem for the small ruminant industry. As gastrointestinal nematodes resistance to anthelmintics prevails, cost to the rearing of sheep also. Persistent evaluation of the DBR1 locus and its transcriptome will help us to better understand its role in disease resistance or susceptibility to *H.contortus* and can be used as one of the genetic marker for selection of disease resistant breeds or animals. Anthelmintic resistance is a big problem for livestock producers and demand for production of organic products is gaining momentum across the world. Hence, selection of resistant animals on genetic basis could be a better substitute for these problems. It's an initiative in this field and further research is required for better understanding of nematode resistance in sheep and assessment of other related genes.

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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 and approval for collection of blood sample from sheep was taken from IAEC vide agenda item No. 16 dated 16.01.2013.

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Nanotechnology Applications and Potential in Parasitology: An Overview

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ABSTRACT

Nanotechnology is an emerging field of science which deals with synthesis, strategy and manipulation of particle's size and structure for a variety of applications. Therapeutic application of nanomedicine involves use of molecular tools in the development of various types of drug-carrier nano-devices as targeted delivering for control of infectious diseases of various etiologies. Among other applications, improved means of diagnosis and development of adjuvants for vaccination are also relevant. The use of nanotechnology in parasitic diseases is expected to open new opportunities by overcoming the increasing problem of drug resistance in several parasites by providing more effective and safer alternatives to traditional therapy. Researchers have indicated that among various nano-materials, oxidized metals particularly silver and gold have growth inhibitory or cytotoxic effect on several protozoans like *Plasmodium*, *Leishmania* and *Toxoplasma* and helminth parasites including *Echinococcus*, *Fasciola* and *Schistosoma*. Nanoparticles (NPs) in combination with current drugs have been used as anti-protozoan and anthelmintic activates. In green synthesis, some bio-active plant extracts have shown good potential for effective and safe control of arthropod vectors of disease and other parasites. In diagnosis of parasitic disease, the concept of NPs conjugation with the parasite biomarkers, has been shown sensitive in the early detection of especially important protozoan diseases. NPs have been shown to act as vaccine candidates against toxoplasmosis and malaria, and as adjuvant to improve immune response against schistosomiasis, visceral leishmaniasis and coccidiosis. Their utility as a vaccine delivery system in parasitic diseases is also being investigated.

Key words: Nanotechnology, Nanoparticles, Nanomedicine, Parasitic diseases, Drug delivery systems, Diagnosis, Vaccine.

Introduction

Nanotechnology is an emerging field of science which involves a multidisciplinary approach for the development of innovative products on a nano-scale for an array of applications. The concept of nanotechnology is recent and its application to the prevention and treatment of diseases as well as for diagnostic purposes (nanomedicine) is in evolutionary stage. As applied to human and veterinary therapeutics, nanomedicine involves use of molecular tools in the development of various types of drug-carrier nano-devices as new strategies for targeted delivery (Irache *et al*., 2011). Various types of nano-materials in medicine that have been developed and characterized, include drug nanocrystals, polymer therapeutics, lipid-nanosized and polymeric-nanosized drug delivery systems. Metallic nanoparticles (NPs) such as silver and gold have been considered most promising nanomaterials. Other elements like iron and silica have also been used due to remarkable anti-microbial properties while certain polysaccharide like chitosan (CS) has good properties as a drug delivery system. Nanoparticulate delivery systems have found various applications in nanomedicine: imaging, cancer targeting, detection and control of infectious diseases, with higher specificity and lower side effects (Lin *et al*., 2014).

Parasitic diseases affect millions of people worldwide especially in developing countries and their treatment involves challenges such as drug resistance. Nanotechnology applications in parasitology mainly focus on three main aspects

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viz. diagnosis, treatment and vaccination. The development of new drug delivery systems favouring longer dose intervals and the increase in drug activity against parasites are some of the major issues studied in the applications of nanotechnology in parasitology. One of the most important problems in medical parasitology research as well as the veterinary counterpart is the development of drug resistance. Nanoparticles may offer a potential tool to reduce the risk of resistance to traditional drugs by avoiding some of the resistance mechanisms, increasing bioavailability and potentiating the target of the treatment. Nanoparticles can help in the mass treatment of ectoparasites by facilitating the drug-parasite interaction. Benefit of synthesis of metal NPs using plant extracts (green synthesis) is that it is economical, energy-efficient, cost-effective, provides healthier work places and communities by protecting human and animal health through safe products. There is a vast reservoir of medicinal plants with documented activity against parasitic protozoa (Chhabra *et al*., 2014a), helminths (Pathak and Chhabra, 2014) and arthropods (Chhabra *et al*., 2014b) whose action can be enhanced by the process of green synthesis. The application of NPs for development of diagnostic methods in parasitic diseases has also gained importance in the last decade. The concept of NPs conjugation with the parasite biomarkers such as heat shock proteins, polyclonal IgG antibodies or oligonucleotide probes, has been used to achieve rapid and sensitive diagnosis of various pathogenic parasites. In vaccination, nanotechnology has offered alternatives to produce new prophylactic candidates, adjuvants and delivery systems which are more convenient to use, safer and easy to administer. An overview of the possible nanotechnology applications in parasitic diseases is presented hereunder with main focus on treatment highlights from recent research.

Diagnosis

Among metallic nanoparticles, gold (Au) NPs have a significant role in the diagnosis of various parasitic infections. In the diagnosis of malaria, Au NP-based fluorescent immunoassay for antigen detection (Guirgis *et al.*, 2012), specific calorimetric aptasensor based on cationic polymers and Au NPs (Jeon *et al.*, 2013) and DNA aptamer (selected oligonucleotide molecules from *Plasmodium falciparum* DNA) conjugated to Au NPs (Cheung *et al.*, 2013) were used to achieve early and rapid diagnosis of malignant malaria. A rapid diagnostic kit linked *P. falciparum* biomarkers *viz.* histidine-rich protein and pan-*Plasmodium* lactic hydrogenase with Au NPs in an immuno chromatographic assay for diagnosis of malaria in clinical samples was developed (Wu *et al.*, 2014). Highly sensitive magnetic NPs PCR enzyme-linked gene assay (MELGA) was developed for the specific detection of gametocytes of *P. falciparum in vitro* studies (Tangchaikeeree *et al*., 2017). For diagnosis of amoebiasis, fluorescent silica NPs (FSNP) conjugated with monoclonal anti-*E.histolytica* IgG 1 showed high sensitivity and specificity (Hemadi *et al.*, 2015). In leishmaniasis, Au NPs conjugated with four oligonucleotide probes, targeting DNA of *Leishmania* kinetoplastid, were used (Andreadou *et al.*, 2014). A piezoelectric immunoagglutination assay for *Toxoplasma gondii* antibodies using Au NPs was evaluated (Wang *et al.*, 2004). Aly *et al*. (2018) demonstrated that bioconjugated silica-coated NPs (SiO_2NP) with polyclonal antibodies (pAb) increased the sensitivity and specificity of nano-sandwich ELISA for detection of *T.gondii* antigens in serum and urine samples of man in active (early) and light infections. Oligonucleotide-Au NPs networks for the molecular detection of *Cryptosporidium parvum* heat shock protein-HSP 70 mRNA, was considered to be a novel strategy (Javier *et al.* (2009). An amplification-free molecular assay using oligonucleotide functionalized Au NPs, for detection of *C. parvum* oocysts in stool was developed (Weigum *et al.*, 2013). The authors opined that this approach offers unique advantages for such Au NPs aggregation assays to be extended towards use in resource-limited areas where protozoan detection is most needed. The synthesized aminopropylsilane (APS)-modified iron oxide (Fe_2O_3) NPs showed high efficiency as an MRI contrast agent for evaluation of liver damage induced by *Opisthorchis felineus* (Demin *et al.*, 2016). Nanobodies, camelid-derived single-domain antibodies specific for *Taenia solium* cysticercosis, were tested in a sandwich ELISA with cyst fluid antigen, and found a promising tool for species-specific diagnosis (Deckers *et al.*, 2009). Au NPs labeled-antigen for early detection

of antibodies of hydatid cyst was developed by Jahani *et al*. (2014).

Treatment

Protozoa

Among diseases caused by protozoan parasites, targeting infected macrophages with NPs has been validated as effective treatment of visceral leishmaniasis (Kunjachan *et al*., 2012). Engineered poly (lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) nanoparticles as carriers of amphotericin B for treatment of leishmaniasis showed promise in developing a liposomal formulation (Nahar and Jain, 2009, de Carvalho *et al*., 2013) but may have safety concerns. On the other hand, silver nanoparticles (Ag NPs) may provide promising lead towards development of cost-effective and safer alternative treatment against cutaneous leishmaniasis (Allahverdiyev *et al*., 2011) and the visceral form of the disease (Zahir *et al*., 2015). As drug delivery system, CS proved suitable for doxorubicin (Kunjachan *et al*., 2011), amphotericin B (Asthana *et al*., 2013) and rifampicin (Chaubey and Mishra *et al*., 2014). Combination of NPs with drugs was observed to enhance the therapeutic efficacy and reduce the toxicity of anti-leishmanial drugs like artemisinin (Want *et al*., 2015) and meglumine antimoniate (Abamor *et al*., 2017). Recently Tiwari *et al*. (2017) reported nanotized curcumin as an adjunct to the standard drug miltefosine for treatment of experimental visceral leishmaniasis. Sousa-Batista *et al*.(2017) demonstrated that lipid-core NPs enhanced the efficacy of the oral drug, quercetin in cutneous leishmaniasis at a very lower dosage level.

Development of a nanoparticulate formulation of diminazine to treat African trypanosomiasis has been reported (Kroubi *et al*., 2010). According to Adeyemi and Whiteley (2013), the enzyme arginine kinase which is critical to the survival of trypanosomes is inhibited by interaction with Ag and Au NPs. The inhibitory interaction could thus be effective treatment of trypanosomiasis (*T.brucei*). A drug nanocarrier based on polyethylene glycol (PEG) covalently attached to PLGA to generate NPs, was coupled to a single domain heavy chain antibody fragment (nanobody) that specifically recognizes the surface of *T. brucei*. Diminazene aceturate encapsulated into liposomes showed *in vitro* and *in vivo* significant results in treatment of *T. evansi* trypanosomiasis (Oliveira *et al*., 2014). NPs loaded with pentamidine as formulation was found effective therapy in both *in vitro* assay as well as *in vivo* murine model in 7-fold and 10-fold lower dose than the minimal curative dose of free pentamidine (Arias *et al*., 2015). In *T. evansi* infection, Manuja *et al.* (2014; 2016) amply demonstrated the enhanced trypanocidal activity of quinapyramine sulfate-loaded sodium alginate nanoparticles (QS-NPs) after conducting *in vitro* safety and toxicity trials by metabolic assay in Vero cell lines, and *in vivo* by mice inoculation. More recently, nerolidol-loaded nanospheres were shown to prevent hepatic stress of mice experimentally infected with *T. evansi* (Baldissera *et al*., 2017).

Silver, chitosan and curcumin as NPs were validated for their anti-*Giardia* activity in infected rats (Said *et al.*, 2012). While combination of nano-Ag and nano-CS gave very good results, the highest effect and complete cure of was gained by combining all three nanoforms indicated above. Synthesis and application of novel graphene oxide (GO) and carbon nanotube (CNTs) oxide composite suggested that the water-dispersible nanomaterial was promising therapeutic delivery agent for bovine trichomoniasis due to *Tritrichomonas foetus* (Zanin *et al*., 2014). Saad *et al*. (2015) proved that the viability of amoebic cysts was highly reduced by a new nanoform treatment with Cu-NPs. The use of chitosan oligosaccharide-functionalized/coated iron oxide NPs for efficient capture and removal of pathogenic *Entamoeba histolytica* cysts from contaminated water was reported (Shukla *et al*., 2015). Ahamed *et al*. (2017) confirmed the antiparasitic activity of Ag-NPs by the significant reduction of trophozoites compared with that of metranidazole, the drug of choice.

Antisense oligonucleotide (AS-ON) encapsulated in NPs evaluated against *Plasmodium falciparum* improved its internalization by infected erythrocytes as well as a significantly stronger inhibitory effect on the parasites than the free compound (Foger *et al*., 2006). Poly-ε-caprolactone nanocapsules (PCL) NPs loaded with halofantrine (Leite *et al*., 2007) and PCL

nanocapsules to deliver the classical antimalarial quinine have proved effective against *P.berghei* (Hass *et al*., 2009). Evaluation against *P.berghei* in infected rodents indicated that binding of curcumin (Akhtar *et al*., 2012) and chloroquine (Tripathy *et al*., 2013) to CS increased chemical stability and enhanced bioavailability. Ag NPs green synthesized using extract of leaves of *Andrographis paniculata*, *Pteridium aquilinum* (Panneerselvam *et al*., 2011, 2016), *Catharanthus roseus* (Ponarulselvam *et al*., 2012) and *Azadirachta indica* (Murugan *et al*., 2016) demonstrated potential for antiplasmodial activity against *P. falciparum*. Solid lipid NPs (SLN) loaded with buparvaquone (BPQ) for targeted delivery in theileriosis was found promising approach for targeted and improved delivery (Soni *et al.*, 2014).

Evaluation of low levels of Ag NPs in drinking water of broilers as potential replacement of antibiotic coccidiostat (salinomycin or baycox) on parameters such as weight gain, lesion score and faecal egg count (Chauke and Siebrits, 2012) gave inconclusive results. In contrast, an investigation (Dkhil *et al.*, 2015a) on anticoccidial properties of zinc oxide (ZnO) NPs on *Eimeria papillata*-induced infection in mice, found protective effects of the treatment.

Ag NPs evaluated singly or combined with CS displayed promising anti-toxoplasmic potential (Gaafar *et al.*, 2014). The animals that received these compounds showed statistically significant decrease in the mean number of parasitic count in the liver and spleen, when compared to the corresponding control group. The peritoneal exudates of treated animals demonstrated stoppage of movement and deformity in shape of trachyzoites under light microscope whereas by scanning electron microscope, the organisms appeared mutilated. Moreover, gamma interferon was increased in the serum of animals receiving these compounds, providing the effectiveness of NPs against experimental *Toxoplasma* infection. Inorganic NPs of Au, Ag and platinum (Pt) demonstrated inhibitory activity against *T. gondii* via changes in redox status and mitochondrial membrane potential (Adeyemi *et al*., 2017). Polyvinyl alcohol conjugated with CS was proven to suppress the attachment of *Cryptosporidium* sporozoites to enterocytes *in vitro* (Luzardo *et al*., 2012). The viability of C. *parvum* oocysts was highly reduced in Ag NPs treated group (Saad *et al*. 2015, Cameron *et al*., 2016). Treatment with encapsulated nitazoxanide in SLN considerably reduced cysts on 6th day than the drug used in free form (Sedighi *et al.*, 2016).

Helminths

In helminth parasites, Ag NPs synthesized with the soil-isolated fungus *Trichoderma harzianum* combined with the anthelmintic triclabendazole enhanced the anti-*Fasciola* (*F.hepatica*) properties in terms of increased percentage of non-hatching eggs (Gherbawy *et al.*, 2013). Praziquantel (PZQ) in a nano carrier, nano-encapsulated curcumin (NEC), used for experimental *Opisthorchis viverrini* infection in hamsters gave encouraging results in terms of combining anti-oxidative effects of curcumin with the standard anthelmintic value of PZQ (Charoensuk *et al*., 2016). The studies of Khan *et al.* (2015) on ZnO NPs *in vitro* on *Gigantocotyle explanatum* of Indian water buffalo reflected strong anthelmintic response indicating pronounced tegumental damage with the disruption of surface papillae and annulations, attributable to the production of reactive oxygen species that target a variety of macromolecules such as nucleic acid, protein and lipids. Anti-schistosomal and antioxidant role of Au NPs against hepatic (Dkhil *et al.*, 2015b) and splenic damage (Dkhil *et al.*, 2017) and protective role of selenium (Se) NPs against hepatic injury (Dkhil *et al.*, 2016) in *Schistosoma mansoni* infected mice have been demonstrated. Clinical study conducted by Eissa *et al.* (2015) was suggestive that miltefosine incorporated lipid nanocapsules (MFS-LNC) was a potential single dose oral nanomedicine for enhanced therapy of *S. mansoni*. El-Feky *et al.* (2015) improved the biological characteristics of antischistosomal drug PZQ by incorporating it into montmorillonite (MMT) clay nanoformulation as a delivery carrier which enhanced drug efficacy against murine *S. mansoni* infection.

Anthelmintic efficiency of Au NPs synthesized by treating the mycelia-free culture filtrate of the phytopathogenic fungus, *Nigrospora oryzae* was

tested against poultry cestode, *Raillietina* sp. (Kar *et al*., 2014). The enhancement of therapeutic efficacy of PZQ against *Echinococcus granulosus* natural infection in dogs by loading with hydrogenated castor oil solid-lipid NP suspension, was demonstrated in terms of stool-ova reduction and complete elimination of the tapeworm (Xie *et al*., 2011). Studies on the *in vitro* scolicidal effect of SE NPs bio-synthesized by newly isolated marine bacterial strain *Bacillus* sp. MSh-1 (Mahmoudvand *et al*., 2014) and aqueous aerial extract of the fungus *Penicillium aculeatum* (Rahimi *et al*., 2015) against protoscolices of *E. granulosus,* proved that these biogenic materials have potential scolicidal effects. *In vitro* anthelmintic studies on ZnO and FeO NPs against *Toxocara vitulorum* showed that NPs exert their effects v*ia* induction of oxidative/ nitrosative stress resulting significant decrease in worm mobility and increased mortality rate (Dorostkar *et al*., 2017). Albendazole bound to CS as microspheres, was found effective in the treatment of visceral larva migrans caused by *T. canis* (Barrera *et al*., 2010). Studies on SLN of albendazole in *T. canis* infection in mice confirmed the promising anthelmintic efficacy with reduction in larval count in liver, lung, brain and kidney (Kudtarkar *et al*., 2017). *In vitro* anthelmintic assay of essential oils of nanostructured *Melaleuca alternifolia* and of terpinen-4-ol confirmed ovicidal and larvicidal activity of *Haemonchus contortus* (Grando *et al*., 2016). Evaluation of *in vitro* anthelmintic activity of Ag Nps synthesized aqueous extract of *Ziziphus jujuba* and *Azadirachta indica* leaf extract against the gastro-intestinal nematode (GIN), *H. contortus* revealed potent anthelmintic properties (Preet and Tomar, 2017, Tomar and Preet, 2017). The use of *Eucalyptus staigeriana* essential oil nano-emulsion as phytotherapy combined with FAMACHA as targeted selective treatment (with levamisole) for sustainable control of GIN in small ruminants indicated that this approach can be an alternative to minimize the use of synthetic anthelmintics, and to control resistant GIN populations of small ruminants (Ribeiro *et al*., 2017). Singh *et al.* (2012) reported that nanosilver at relatively low concentrations acted as a potential drug-adjuvant to the current diethylcarbamazine citrate (DEC) therapy for its effective cidal activity against *Brugia malayi* microfilariae. Green synthesized Ag Nps from *Acacia auriculiformis* at lower dose were shown to exhibit antifilarial activity through regulation of reactive oxygen species (ROS) mediated apoptosis against the bovine filarial parasite *Setaria cervi* (Saini *et al.* 2016).

Arthropods

Arthropods, apart from being injurious to human and animal health directly, include vectors of important diseases. Growing resistance to chemical insecticides having eroded their efficiency, there is an active search for alternative means of control. A wide selection of the plants has been found to contain active ingredients with proven toxicity for arthropods, consistent with safety concerns for non-target organisms, environment, and human health. Extracts or essential oils from a large number of plants screened for their insecticidal or acaricidal activity, constitute a rich source of bioactive compounds potential candidates for combining with metallic NPs via “green synthesis” are summarized. Such green synthesized NPs have great potential as a tool for control of arthropods. Some of the plants screened by biosynthesis route, in particular against mosquito vectors of malaria and filaria (Table 1) and other harmful insects (Table 2) as well as ixodid ticks (Table 3) are tabulated.

Table 1: Potential effectiveness of green synthesized nanoparticles (NPs) against vectors mosquitoes

NPs	Plant species used for aqueous extract	Plant parts	Mosquito instars	LC50 mg/L	Reference
A: *Culex quinquefasciatus*					
Ag	*Rhizophora mucronata*	Leaf	Larva	0.89	Gnanadesigan *et al*. (2011)
Ag	*Tinospora cordifolia*	Leaf	Larva IV	6.96	Jayaseelan *et al*. (2011)
Ag	*Eclipta prostrata*	Leaf	Larva IV	4.56	Rajakumar and Rahuman (2011)
Ag	*Nelumbo nucifera*	Leaf	Larva IV	1.10	Santhoshkumar *et al*. (2011)
Ag	*Annona squamosa*	Leaf	Larva I-IV Pupae	0.04-0.41 0.79	Kumar *et al*. (2012)
Au	*Anthocepholus cadamba*	Leaf	Larva III	1.08	Kumar *et al*. (2013)
Ag	*Drypetes roxburghii*	Fruit	Larva II –IV	0.86-1.28	Halder *et al*. (2013)
Ag	*Morinda tinctoria*	Leaf	Larva III	1.44	Ramesh Kumar *et al*. (2014b)
Ag	*Cinnamomum zeylanicum*	Bark	Larva I Larva II-IV	100% death 1.5-4.0	Soni and Prakash (2014a)
Ag	*Azadirachta indica*	Leaf & bark	Larva I-II Pupa Adult	100% death 4.00 1.06	Soni and Prakash (2014b)
Ag	*Sterculia foetida*	Seed	Larva	4.13	Rajasekharreddy and Rani (2014)
Ag	*Caulerpa scalpelli-formis* (sea weed)	Frond	Larva I-IV Pupae	3.08-5.86 7.33	Murugan *et al*. (2015)
Ag	*Azadirachta indica*	Leaf	Larva	0.047	Poopathi *et al*. (2015)
Ti	*Mangifera indica*	Leaf	Larva IV	4.34	Rajakumar *et al*. (2015)
Ag	*Cassia hirsuta*	Leaf	Larva II-IV	4.43	Adesuji *et al*. (2016)
Ag	*Leucas aspera*	Leaf	Larva	5.06	Elumalai *et al*. (2017)
Ag	*Hyptis suaveolens*	Leaf	Larva	3.52	Elumalai *et al*. (2017)
Zn	*Momordica charantia*	Leaf	Larva	4.87	Gandhi *et al*. (2017)
Ag	*Polianthus tuberosa*	Bud	Larva III Larva IV	9.65 7.94	Rawani (2017)
B: *Anopheles stephensi*					
Ag	*Nelumbo nucifera*	Leaf	Larva IV	1.10	Santhoshkumar *et al*. (2011)
Ag	*Annona squamosa*	Leaf broth	Larva I-IV Pupa	0.16-2.12 3.74	Kumar *et al*. (2012)
Ag	*Musa paradisiaca*	Leaf	Larva IV	1.39	Jayaseelan *et al*. (2012)
Ag	*Drypetes roxburghii*	Fruit	Larva II-IV	0.73-0.928	Halder *et al*. (2013)
Ag	*Solanum nigrum*	Leaf & berry	Larva	1.33-1.56	Rawani *et al*. (2013)
Ag	*Plumeria daemia*	Latex	Larva I-IV	4.41-6.47	Patil *et al*. (2012a)
Ag	*Plumeria rubra*	Latex	Larva II Larva IV	1.10 1.74	Patil *et al*. (2012b)

Ag	*Cinnamomum zeylanicum*	Bark	Larva I-IV	2.0-10	Soni and Prakash (2014a)
Au	*Cinnamomum zeylanicum*	Bark	Larva I Larva II-IV	100% death 1.0-2.0	Soni and Prakash (2014a)
Ag	*Sterculia foetida*	Seed	Larva	3.99	Rajasekharreddy and Rani (2014)
Ag	*Avicennia marina*	Leaf	Larva	4.37	Balakrishnan *et al*. (2016)
Ag	*Azadirachta indica*	Seed kernel	Larva I-IV Pupa	3.9-6.5 8.2	Murugan *et al*. (2016)
Ag	*Leucas aspera*	Leaf	Larva	4.69	Elumalai *et al*. (2017)
Ag	*Hyptis suaveolens*	Leaf	Larva	4.04	Elumalai *et al*. (2017)
Zn	*Momordica charantia*	Leaf	Larva	5.42	Gandhi *et al.* (2017)
C: *Anopheles subpictus*					
Ag	*Tinospora cordifolia*	Leaf	Larva IV	6.43	Jayaseelan *et al*. (2011)
Ag	*Eclipta prostrata*	Leaf	Larva IV	5.14	Rajakumar and Rahuman (2011)
Ag	*Nelumbo nucifera*	Leaf	Larva IV	0.69	Santhoshkumar *et al*. (2011)
Ag	*Musca paradisiaca*	Leaf	Larva IV	1.39	Jayaseelan *et al*. (2012)
Ti	*Solanum trilobatum*	Leaf	Larva IV	1.94	Rajakumar *et al*. (2014)
Ti	*Mangifera indica*	Leaf	Larva IV	5.84	Rajakumar *et al*. (2015)
D: *Aedes aegypti*					
Ag	*Tinospora cordifolia*	Leaf	Larva IV	6.43	Jayaseelan *et al*. (2011)
Ag	*Nelumbo nucifera*	Leaf	Larva IV	0.69	Santhoshkumar *et al*. (2011)
Ag	*Annona squamosa*	Leaf broth	Larva I-IV Pupae	0.02-0.30 0.56	Kumar *et al*. (2012)
Ag	*Pergularia daemia*	Latex	Larva II Larva IV	1.49 1.82	Patil *et al*. (2012a)
Ag	*Plumeria rubra*	Latex	Larva II Larva IV	1.49 1.82	Patil *et al*. (2012b)
Ag	*Pedilanthus tithymaloides*	Leaf	Larva I-IV Pupae	0.03-0.09 0.02%	Sundaravadivelan *et al*.(2013)
Ti	*Solanum trilobatum*	Leaf	Larva IV	1.94	Rajakumar *et al*. (2014)
Ag	*Morinda tinctoria*	Leaf	Larva III	3.63	Ramesh Kumar *et al*. (2014a)
Ag	*Sterculia foetida*	Seed	Larva	4.23	Rajasekharreddy and Rani (2014)
Ag	*Leucas aspera*	Leaf	Larva IV	8.56	Suganya *et al*. (2014)
Ag	*Delphinium denudatum*	Root	Larva II	9.60	Suresh *et al*. (2014)
Ag	*Phyllanthus niruri*	Leaf	Larva I-IV	3.90-8.90	Suresh *et al*. (2015)
Ag	*Azadirachta indica*	Leaf	Larva	0.07	Poopathi *et al*. (2015)
Ag	*Avicennia marina*	Leaf	Larva	7.41	Balakrishnan *et al.* (2016)
Ag	*Leucas aspera*	Leaf	Larva	4.02	Elumalai *et al*. (2017)
Ag	*Hyptis suaveolens*	Leaf	Larva	4.63	Elumalai *et al*. (2017)

Table 2: Green synthesized nanoparticles (NPs) showing potential against insects other than mosquitoes

NPs	Synthesis route	Target insects	Instars	LC50 mg/L	Reference
Ag	*Tinospora cordifolia*	*Pediculus h. capitis*	Adult	12.46	Jayaseelan *et al.* (2011)
Ag	*Lawsonia innermis*	*Pediculus h. capitis*	Adult	1.33	Marimuthu *et al*. (2012)
Ti	*Solanum trilobatum*	*Pediculus h. capitis*	Adult	4.34	Rajkumar *et al*. (2014)
Ti	*Vitex negundo*	*Pediculus h. capitis*	Adult	24.32	Gandhi *et al*. (2016)
Zn	*Momordica charantia*	*Pediculus h. capitis*	Adult	14.38	Gandhi *et al*. (2017)
Ag	*Lawsonia innermis*	*Bovicola ovis*	Adult	1.41	Marimuthu *et al.* (2012)
Ti	*Catharanthus roseus*	*Bovicola ovis*	Adult	6.56	Velayutham *et al*. (2012)
Ag	*Musa paradisiaca*	*Hippobosca maculata*	Larva	2.02	Jayaseelan *et al.* (2012)
Ag	*Cissus quadrangularis*	*Hippobosca maculata*	Adult	6.30	Santhoshkumar *et al.* (2012)
Ti	*Catharanthus roseus*	*Hippobosca maculata*	Adult	7.09	Velayutham *et al*. (2012)
Ag	*Euphorbia prostrata*	*Hippobosca maculata*	Adult	2.55	Zahir and Rahuman (2012)
Ag	*Manilkara zapota*	*Musca domestica*	Adult	3.64	Kamaraj *et al*. (2012)
Zn	*Lobelia leschenaultiana*	*Lucilia sericata*	Larva II	0.78	Banumathi *et al*. (2017)

Table 3: Toxic potential of green synthesized nanoparticles (NPs) against tick vectors

NPs	Plant species used for aqueous extract	Plant parts	Tick instars	LC50 mg /L	Reference
A: *Rhipicephalus microplus*					
Ag	*Mimosa pudica*	Leaf	Larva	8.98	Marimuthu *et al.* (2011)
Ag	*Manilkara zapota*	Leaf	Larva	3.44	Rajakumar and Rahuman (2012)
Ag	*Cissus quadrangularis*	Leaf	Larva	7.61	Santhoshkumar *et al*. (2012)
Ti	*Calotropis gigantea*	Leaf	Larva	5.43	Marimuthu *et al*. (2013)
Ti	*Mangifera indica*	Leaf	Larva	28.56	Rajakumar *et al.* (2015)
Zn	*Lobelia leschenaultiana*	Leaf	Adult	1.7	Banumathi *et al.* (2016)
Zn	*Momordica charantia*	Leaf	Larva	6.87	Gandhi *et al*. (2017)
B: *Hyalomma anatolicum anatolicum*					
Ag	*Ocimum canum*	Leaf	Larva	0.78	Jayaseelan and Rahuman (2012)
Ti	*Solanum trilobatum*	Leaf	Larva	4.1l	Rajakumar *et al*. (2014)
Ti	*Mangifera indica*	Leaf	Larva	33.17	Rajakumar *et al.* (2015)
C: *Haemaphysalis bispinosa*					
Ag	*Musa paradisiaca*	Leaf	Larva	1.87	Jayaseelan *et al*. (2012)
Ag	*Euphorbia prostrata*	Leaf	Larva	2.30	Zahir and Rahuman (2012)
Ti	*Calotropis gigantea*	Leaf	Adult	9.15	Marimuthu *et al*. (2013)
Ti	*Mangifera indica*	Leaf	Larva	23.81	Rajakumar *et al.* (2015)
D: *Hyalomma marginatum isaaci*					
Ag	*Ocimum canum*	Leaf	Larva	1.51	Jayaseelan and Rahuman (2012)

Immunity and Immunization

Several NPs were formulated for vaccine development as they protect DNA vaccines from degradation. The use of chemical component offered further potential help in vaccine development. Since self-assembling polypeptides have the ability to induce CD 8^+ and CD 4^+ T cells, they were used to induce a long-lasting immune response to specific epitope of *P. falciparum* circumsporozoites protein (Kaba *et al.*, 2012) and as immunogenic NP-platform for *Toxoplasma* peptide vaccine (El Bissati *et al.*, 2014). Further to eliminate the problems associated with the use of extraneous adjuvants, Kaba *et al.* (2018) currently designed a self-assembling protein nanoparticle (SAPN) with built-in flagellin domains which increased protective efficacy of *P. falciparum* based vaccine. This new finding will be promising strategy for the development and delivery of a safe vaccine for malaria and other related diseases. Against *Eimeria tenella* cocidiosis in chickens, adjuvant effect of ginsenoside-based NPs (ginsomes) in recombinant vaccine which promoted subunit vaccine to induce a strong immune response and protective effect (Zhang *et al.*, 2012). The results demonstrated that the adjuvant ginsomes can promote sub-unit vaccine to induce protection in terms of survival rate, body weight gains, lesion scores and oocyst counts.

Immunization studies such as Oliveira *et al.* (2012) suggested that the combination of CS NPs associated to the antigen Sm Rho and CPG is an efficient vaccine candidate against *S.mansoni*, by modulating the pathological response of granuloma and also induce protection against infection. As an initiation, new B-cell peptide epitopes based on the aspartic protease of Na-APR-1, conjugated to self-adjuvanting lipid core peptide (LCP) vaccine candidates were developed to combat human hookworm *Necator americanus*. These were able to self-assemble into NPs if administered to mice and generated antibodies that recognized the parent epitope which provided delivery system design for the development of a vaccine (Fuaad *et al.* 2015).

Conclusion and future directions

The overview as above, has attempted to highlight an area of increasing global interest and the recent progress in it. Despite its enormous potential, the application of nanotechnology in parasitology is still in infancy. The harnessing of nanotechnology can be an obvious approach to efficacy enhancement of plant-derived agents, alongside their inherent advantages of safety, sustainability and cost-benefit ratio. Not surprisingly, therefore, the most spectacular application studies thus far, have been in the control of arthropod vectors of disease. Widespread anthelmintic resistance among helminth parasites, especially in the pathogenic GIN like *Haemonchus*, should spur future investigations in seeking solutions through nanotechnology. The aspects of 'diagnosis' and 'vaccine' development via nano-biology, are also largely unexplored in livestock parasites and as such are fertile directions for future research.

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All authors declare that there exist no commercial or financial relationships that could in any way lead to a potential conflict of interest.

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An Overview on Dog Circovirus - A New Canine Pathogen

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ABSTRACT

Dog circovirus (DogCV) is a newly discovered mammalian circovirus (CV), and is the cause of various gastrointestinal problems like diarrhea, vomiting and hemorrhagic enteritis/gastro-enteri-tis in canines. DogCV was detected in various internal organs of domestic dogs and wild canids including wolves, foxes indicating that DogCV prevalence is not only restricted to dogs but is also wide spread in wild canines. Recently, DogCV might act on alimentary canal as a co-factor in disease outcome in canines with multiple infections and may not be detected alone but with other key enteric pathogens like canine parvovirus-2(CPV-2) and canine distemper virus (CDV). In spite of the prevalence of DogCV infection, its pathogenic role is still undetermined in domestic and wild canids. This review article aims to provide information about DogCV morphology, genomic organization, replication, pathogenesis, epidemiology and application of various diagnostic methods.

Keywords: DogCV, Vasculitis, ssDNA, Hemorrhagic enteritis, Co-infection, rolling circle replication

INTRODUCTION

Circoviruses (CVs) are icosahedral viruses, measuring 15–20 nm and having a circular single stranded DNA (ssDNA) of 1.7–2.3 kb (Tischer et al., 1974). Taxonomically, CVs are included in *Circoviridae* family under genus *Circovirus*. CV was detected for the first time in a porcine kidney cell line (PK15) leading to the name porcine circovirus type -1 (Tischer et al., 1974). Followed subsequently by a second porcine circovirus, PCV2 was detected and found responsible for porcine circovirus associated disease (PCVAD) (Meng, 2012; Pearodwong et al., 2015; Shin et al., 2015). CVs were also detected in birds, the most important out of which are psittacine beak and feather disease virus and pigeon circovirus (Todd, 2004). Due to the increasing available techniques and use of deep sequencing techniques various other CV sequences have been identified from environmental specimens and fish, arthropods and amphibians (Blinkova et al., 2009; Lorincz et al., 2012; Rosario et al., 2009, 2011; Tarjan et al., 2014).

For the first time DogCV was identified in canines, in 2012 (Kapoor et al., 2012). It was found in a dog serum from an apparently healthy dog, which later on was suspected to be associated with necrotizing vasculitis and granulomatous lymphadenitis in dogs (Li et al., 2013). Moreover, a CV was recently identified from serum samples of foxes with unexplained neurological signs with the help of viral metagenomics (Bexton et al., 2015).

Viral morphology and physico-chemical properties

DogCV is a non-enveloped, icosahedral, circular, single-stranded DNA virus having a circular genome approximately 2 kb in length and diameter ranging between approximately 15 and 25 nanometers (Thaiwong et al.,2016). CV doesn't hemagglutinate erythrocytes from different animals (Allan et al., 2000).CVs are resistant to pH 3, chloroform, 56°C and 76°C (Allan et al., 2000).

Genome organization

DogCV possesses an ambisense genomic organization with two inversely arranged ORFs which encodes for the rolling circle replication initiator protein gene replicase (Rep) and a capsid protein gene (Cap) respectively, similar to other animal circoviruses (Biagini et al.,2011, Decaro et al., 2014). The viral Rep proteins are

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made up of 303 amino acids and the viral capsid protein of 270 amino acids. The amino terminus of DogCV capsid protein contains a 30-aa-long arginine (R) - rich stretch (Cheung et al.,2012).There are two intergenic non-coding portions of the genome that are made up of 135 and 203 nucleotides, which contains a thermodynamically stable stem-loop for initiation of rolling-circle replication and a characteristic 9-nt sequence, TAGTATTAC (Cheung et al.,2012). The capsid and replicase proteins of DogCV share approximately <25% and <50% identities, respectively, with the other known animal circoviruses (Kapoor et al., 2012). The 203-nt-long intergenic noncoding region of DogCV was found to share 91% nucleotide identity over 150-nt of sequence of pine marten torque teno-virus(Van den et al.,2012) providing the first evidence of an evolutionary relationship between two different virus families that includes genetically diverse viruses with ssDNA circular genomes, *Circoviridae* and *Anelloviridae* (Kapoor et al., 2012).

Viral replication

Rolling circle replication is a method of replication of CVs (Cheung et al., 2012). The replication protein *Rep* is an important protein dimer that needs to be encoded by its own respective plasmid or the virus DNA coded for in the DNA. This replication protein cleaves DNA at specific locations thus exposing a free 3'OH group for viral or cellular polymerase to act (Cheung et al., 2011). The DNA replication of CV occurs in the nucleus of the cell in which CV enters easily as it is very small enough to pass through (Cheung et al., 2012). The palindrome sequence in DogCV at the origin of replication site is made up of 12-nt pairs (stem) at the origin of replication site and an open loop of 10-nt (CATAGTATTA) (Cheung et al., 2012).

Pathogenesis

The pathogenic role of DogCV in domestic and wild canids is still not very well understood and further studies are needed in order to understand the pathogenesis of this virus. In domestic and wild canids viral and bacterial infections, plays a major role in complicating pathogenesis of DogCV, as it do in cases of PCV2 (Opriessnig and Halbur, 2012). Studies have also shown that DogCV seems to show a tropism for particular tissues like lymphatic tissues (Li et al., 2013) at least in case of dogs, wolves and badgers. DogCV was detected with higher titers in some tissues like spleen and lymph nodes tissues as compared to other tissues thus compromising the immune status of the host (Zaccaria et al., 2016). The viral inclusions and multinucleate giant cell formation are two distinct histological features of PCV2 infection neither of which was detected by routine histology in the dogs infected with DogCV (Segales et al., 2002). However, by electron microscopy, macrophages within the lymph node having abundant cytoplasmic viral inclusions composed of dense granular or paracrystalline arranged virus could be detected (Li et al., 2013). DogCV DNA was also detected in various other lymphoid tissues, including Peyer's patches. DogCV DNA was detected even from dogs showing no clinical or histological enteric lesion and the DNA of DogCV was also found in small, end-capillary endothelial channels of the intestinal lamina propria and the adrenal cortex; however (Li et al., 2013). This implies that the virus can invade various body tissues with or without resulting pathogenesis, which needs more detailed investigations.

Epidemiology

CVs have been detected in several animal species like birds, pigs, dogs and including many wild animal species like wolves, foxes and badgers (Biagini et al., 2011; Kapoor et al., 2012; Todd, 2004). Among wild animals, DogCV was detected in wolves and badgers but not concurrently as in dogs. The geographical distribution of DogCV at present seems to depend on the type of host species weather domestic or fee ranging as well as the properties of the virus and prevailing environmental conditions of geographical (Zaccaria et al.,2016). More studies are needed in order to find out the role of reservoir and carrier animals needed for the epidemiology and transmission among susceptible population of DogCV.

Symptoms

According to the studies reported in domestic dogs, the symptoms observed in DogCV infection includes vomiting, diarrhea (may or may not be bloody), lethargy, thrombocytopenia, neutropenia and vasculitis, hemorrhages among various organs involving intestinal tract like liver, kidney and lymph node (Li et al., 2013).Studies have also shown that the target organs affected varied even in this small set of animals, but necrotizing vasculitis and hemorrhage were common and observed in all dogs leading to the conclusion that DogCV probably infect the endothelial cells (Li et al., 2013).The dogs found positive by In-situ hybridization (ISH), the disease signs varied, and clinical, gross, and microscopic features in some of the disease symptoms were similar to those associated with PCV2 infection (Li et al., 2013). Co-infection is also a common occurrence in animals afflicted with DogCV. CDV and CPV-2 infections were the most common co-infections in addition to DogCV infection of domestic dogs and other wild canids (Zaccaria et al., 2016). It is also suspected that young ones may be more at risk of developing life-threatening symptoms than adult animals; however this aspect has not yet been scientifically proved (Zaccaria et al., 2016).

Diagnosis

1. **Detection of the Virus**

a. **Electron microscopy:** Electron microscopy has been used to find out the presence of the virusin the histological specimen that can obviate the need for virus isolation or serology. Formalin-fixed affected tissues from the sentinel dog affected with DogCV infection was re-fixed in 2.0 % glutaraldehyde and processed and embedded in epoxy resin (Li et al., 2013). Thick sections of the affected tissues were stained with toluidine blue and ultra thin sections from affected areas of the tissues were examined by using a transmission electron microscope (Li et al., 2013). Ultrastructural analysis of the tissue showing lesions, from affected dogs revealed macrophages along with large numbers of intra-cytoplasmic inclusions. Inclusions found were round, irregular, <0.5µm in size and usually clustered (up to 25 per cell) within the cytoplasm. Most of inclusions were granular, electron-dense and had a distinct periphery, some of them contained paracrystalline arrays of icosahedral virions of diameter 9–11µm (Li et al., 2013).

b. **Virus isolation:** Positive samples from DogCV cases were inoculated into different cell lines that support replication of other canine viruses, i.e. canine fibroma (A-72, ATCC CRL-1542), Madin Darby canine kidney (MDCK, ATCC CCL-34), African green monkey kidney (VERO, ATCC CCL-81), Walter Reed canine cells (WRCC), Crandell feline kidney (CRFK, ATCC CCL-94), and Felis catus whole fetus (FCWF, ATCC CRL-2787) (Li et al., 2013). Attempts to cultivate of DogCV were also carried out using freshly-trypsinized cells (Li et al., 2013). The infected cells were monitored for the appearance of cytopathic effects (CPE) and, after 5 days period of incubation, the inoculated cells were tested for DogCV with the help of RT-PCR (Li et al., 2013). The cells were also sub-cultured every 6–8 days for 5 consecutive passages (Li et al., 2013). All these virus isolation attempts using various types of cell lines were however unsuccessful (Li et al., 2013).More studies on the primary canine and/or other cells would be able to provide a better understanding about the culture requirements of the virus.

2. **Detection of viral genome**

a. **Real-time PCR:** Real-time PCR (RT-PCR) assays with specific primers and hydrolysis probe were used to detect DogCV in DNA extracts from dogs sample (Li et al., 2013). Real-time PCR conducted by using the real-time PCR instrument Light Cycler with thermocycling conditions of 10 min at 95°C, 45 cycles of 10 s each at 95°C, 10 s at 51°C and 10 s at 72°C and positive results were obtained (Li et al., 2013).

b. **In-situ Hybridization Analysis:** Tissues were taken from necropsy cases of dogs whose clinical signs or histological lesions matched with the sentinel animal affected

with DogCV infection (i.e., hemorrhagic diarrhea and vasculitis) and ISH was performed on these tissues(Li et al*.,* 2013). On ISH analysis, abundant cytoplasmic viral nucleic acid was detected within germinal centers in macrophages and sub-capsular and medullary sinuses of various lymphoid tissues of the affected dogs (Li et al., 2013).

3. **Detection of Viral Antigen/Antibody**

a. **Serological Tests:** Serological tests for detection of virus specific antibodies are important tool of diagnosis of viral infection. In case of infections with new or recently identified viruses for which prevalence of infections, age at which infection is occurring and the range of susceptible host species is unknown, serological tests prove to be very beneficial (Alistair et al., 2006). Knowledge of these are very important for disease control strategies, application of serological tests helps in formulating such control strategies against new viruses (Alistair et al., 2006). Till date, there have been no serological tests documented in the literature for identification of DogCV antigen or antibody from the serum sample of the affected or healthy canines. As in case of other circoviral infections immunofluoresence and immunoperoxidase tests can be tried to identify the virus antibody and antigen presence (Olvera et al., 2001). A long term study on infection with DogCV would enable us to establish specific and sensitive serological tests in the future.

Treatment

A cure for DogCV infection is currently unknown and there is no specific treatment against it. However, symptomatic treatment based upon the type of symptoms can be done. Symptomatic treatment with steroid, broad spectrum antibiotic, fluid and electrolyte may save the life of the affected animal in single or multifactorial infection.

Prevention and Control

Currently there is no specific vaccine developed against DogCV. DogCV was found in both healthy and diarrheic dog cases (7.3% and 20.1%), its prevalence found higher in dogs having diarrhea (Gentil M et al., 2017). The best prevention for DogCV infection is protecting dogs during the period when maternal immunity wanes off. As no reservoir (animal or environment) for the virus are currently known and as in cases with PCV infections, this virus is also assumed to be fairly resistant to environmental conditions and ubiquitous, therefore further studies in this aspect are wanted.

Conclusion

DogCV is a newly discovered cause of morbidity and mortality in dogs that is often manifested by vomiting and diarrhea with a variety of other non-specific signs and symptoms. However, in many clinical cases of DogCV infection, presence of necrotizing vasculitis and hemorrhages have also been found associated with other disease syndromes along with different etiological agents. DogCV has also been reported in both healthy and diarrheic canine population. Additionally the detection of DogCV in wild canids indicates that the virus remains entrenched in the wild population too.The diagnosis of this new infection is still in its infancy with molecular techniques like RT-PCR reported to be promising. The treatment of this infection is symptom specific while requirement for a vaccine for controlling this infection is still debatable as the exact pathogenic role or reservoirs of this virus are yet to be determined. Further research is required to find out the exact role of this virus as a true etiological agent in dogs and easy and quick means of diagnosis at the field level.

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Satheshkumar S, Asokan SA, Brindha K, Kathiresan D, Kumanan K. Angiogenic characterization of follicular and luteal structures in crossbred cattle using colour Doppler imaging ultrasonography. In: Proceedings of the National Symposium: Addressing animal reproductive stresses through biotechnological tools. Khanapara, Assam, India; 2012. pp. 248.

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