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Lead Guest Editor – Balamurugan Vinayagamurthy

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Editorial – Guest Editors

Special Issue on: Advances in Diagnosis, Prevention, Control and Impact of Animal Diseases.

Dear Readers,

I am delighted to bring out the special issue on Advances in Diagnosis, Prevention, Control and Impact of Animal Diseases (ADPCIAD). I would like to thank guest editors Dr. Gnanavel Venkatesan, Dr. M. Nagalingam, Dr. M. Saminathan and Dr. P. Perumal for their significant contributions to make this special issue with success. I also take opportunity to thank all peer reviewers of this issue for their constructive comments and excellent review. I am grateful to authors of the manuscripts of this special issue for their contributions and timely revisions.

In this special issue, focus is given on molecular diagnostic techniques, impact and control of economically important livestock and zoonotic diseases. It also includes manuscripts on production improvement in livestock along with case studies. I hope readers will get useful information on latest developments and advances in livestock health and production.

I also thank Managing Editor and Management committee of JEBAS journal on my and guest editors behalf for providing us the opportunity and cordial cooperation for bringing out this issue and special thanks are due for Dr Kuldeep Dhama for his motivational effort for the successful release of this special issue.

Thanks to all of you

Balamurugan Vinayagamurthy

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October, 2016

Welcome Message - Managing Editor

(Dr Kamal Kishore Chaudhary, M.Sc, Ph.D)

Dear Authors,

It is with much joy and anticipation that we celebrate the launch of special issue, Spl 3 – ADPCIAD, (Volume 4) of Journal of Experimental Biology and Agricultural Sciences (JEBAS). On behalf of the JEBAS Editorial Team, I would like to extend a very warm welcome to the readership of JEBAS. I take this opportunity to thank our authors, editors and anonymous reviewers, all of whom have volunteered to contribute to the success of the journal. I am also grateful to the staff at Horizon Publisher India [HPI] for making JEBAS a reality.

JEBAS is dedicated to the rapid dissemination of high quality research papers on how advances in Biotechnology, Agricultural sciences along with computational algorithm can help us meet the challenges of the 21st century, and to capitalize on the promises ahead. We welcome contributions that can demonstrate near-term practical usefulness, particularly contributions that take a multidisciplinary / convergent approach because many real world problems are complex in nature. JEBAS provides an ideal forum for exchange of information on all of the above topics and more, in various formats: full length and letter length research papers, survey papers, work-in-progress reports on promising developments, case studies and best practice articles written by industry experts.

Finally, we wish to encourage more contributions from the scientific community and industry practitioners to ensure a continued success of the journal. Authors, reviewers and guest editors are always welcome. We also welcome comments and suggestions that could improve the quality of the journal.

Thank you. We hope you will find JEBAS informative.

Dr. Kamal K Chaudhary
Managing Editor - JEBAS
October 2016

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MULTIPLEX-PCR TO DETECT PATHOGENS AND ANALYSIS OF RELATION OF AGE AND STAGE OF LACTATION OF COWS TO SUB-CLINICAL MASTITIS

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KEYWORDS

Sub-clinical mastitis

Somatic cell count

Multiplex-PCR

Predominant bacteria

Age groups

Stage of lactation

ABSTRACT

In this study, 225 milk samples were collected sequentially during 1st to 88th day from 25 HF cross cows in an organized farm. First five collections were obtained at a weekly interval (1,7,14,21 and 28 days) and later, fortnightly for two months (43, 58, 73 and 88 days). These milk samples were screened for Subclinical mastitis (SCM) by Somatic Cell Count (SCC). Further, multiplex-PCR for detection of *S.aureus*, *E.coli*, *S.agalactiae*, *S.dysgalactiae* and *S.uberis* was employed to detect the major bacterial pathogens. The SCM positivity was assessed based on criteria of SCC ≥ 500,000 cells /ml. The study revealed the high prevalence of variable SCM pattern in milking cows by SCC (73.33 %) in sequentially collected milk samples over a period of 88 days. No specific pattern of prevalence of SCM was observed during the study period. The prevalence of SCM was not influenced by the stage of lactation. In all the stages of lactation and age groups *S. aureus*, Streptococci and *E.coli* were detected with the predominance of *S. aureus*. The varied distribution of organisms in different stages of lactation did not influence the prevalence of SCM. Further, the high prevalence of SCM was noticed in aged cows. Among these, maximum number of milk samples (46 %, 52/113) revealing the presence of pathogens were obtained from cows in the age group 7-11 years. The multiplex PCR was found an easy and rapid method to detect the predominant pathogens causing SCM. The findings emphasize the need to control SCM through sequential monitoring of SCM through SCC, multiplex-PCR and proper managemental practices.

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1 Introduction

The tremendous growth of dairy industry is crippled by bovine mastitis, the most frequent and costly status as it affects the dairy herd worldwide (Halasa et al., 2007). Mastitis is the inflammation of udder parenchyma characterized by pathological changes in the mammary gland as well as physical and chemical changes of the milk. The disease continues to pose a major threat to the animal husbandry and dairy sector. Despite research for several decades, this condition still poses challenge to the researchers. The overall national economic loss in India due to mastitis was to the tune of Rs 7165.51 crores (Bansal & Gupta, 2009). It is a multifactorial disease involving three main elements viz cow, the environment and the pathogen and their complex interaction is incompletely understood (Brand et al., 1996).

Broadly bovine mastitis is of two types, viz. clinical, where the appearance of udder and milk changes and subclinical, wherein the appearance of udder as well as milk is normal. Therefore detection of clinical cases of mastitis (CM) is easy than subclinical form (SCM), which needs application of laboratory tests. The major pathogens responsible for Bovine Mastitis can be further classified as Contagious (*Staphylococcus aureus, S. agalactiae*) and environmental (*Escherichia coli, S. dysgalactiae* and *S. uberis*). Initially, clinical cases could begin as subclinical and hence controlling SCM is the best way to reduce the clinical cases indirectly (Harmon, 1994).

An early diagnosis of mastitis is utmost important to avoid fibrosis of the udder and milk loss. Conventionally, Electrical conductivity (EC), California Mastitis Test (CMT), Somatic Cell Count (SCC) is although rapid ambiguous. The conventional bacterial culturing is cumbersome, time consuming and ambiguous (Hegde, 2011; Nithinprabhu et al., 2013). Of late, the DNA based molecular tools, especially multiplex PCR has been developed and found specific and rapid in detection of major mastitis causing pathogens (Hegde, 2011; Shome et al., 2011).

Bovine mastitis is highly complex disease influenced by various factors, such factors could be analyzed by prospective cohort study based on the sequentially collected data and determines the risk factors. Considering the aforementioned facts, the present study was designed with a focus on Multiplex PCR for detection of predominant pathogens at various time points within a single farm and analyzing the influence of age of cow and stage of lactation with relation to SCM detected by SCC.

2 Materials and methods

2.1 Sampling details

A temporal study was carried out to sequentially monitor the sub-clinical cases of bovine mastitis due to major bacterial pathogens such as *S.aureus, E.coli, S.agalactiae, S.dysgalactiae* and *S.uberis.* In this approach, conventional SCC and Multiplex-Polymerase Chain Reaction (M-PCR) were employed to sequentially monitor the SCM. Furthermore, the effect of stage of lactation and age group of milking cows on prevalence of SCM and in turn influence of SCM on milk production was also investigated. In view of this, the milk samples were sequentially collected from 1st to 88th day. First five collections were collected at a weekly interval (1st ,7th ,14th ,21st and 28th days) and later, fortnightly for two months (43rd , 58th , 73rd and 88th days).

Table 1 Prevalence of SCM in dairy cows by SCC, at different age and stage of lactation.

Days	No. pos/No. tested, percent	Age Groups (years) No. pos/No. tested, percent			Stage of Lactation No. pos/No. tested, percent		
	(1-88th Day)	3-5	5-7	7-11	Early	Mid	Late
1	25/25, 100	7/7, 100	7/7, 100	11/11, 100	15/15, 100	5/5, 100	5/5, 100
7	19/25, 76	6/7, 85.7	4/7, 57.2	10/11, 90.9	13/16, 81.3	4/5, 80.	2/5, 50
14	14/25, 56	5/7, 71.4	2/7, 28.6	7/11, 63.6	7/15, 46.6	3/5, 60.	4/5, 80
21	23/25, 92	7/7, 100	6/7, 85.7	10/11, 90.9	13/14, 92.9	6/6, 100	4/5, 80
28	14/25, 56	3/7, 42.8	3/7, 42.9	6/11, 54.5	8/14, 57.1	4/6, 66.7	2/5, 40.
43	25/25, 100	7/7, 100	7/7, 100	11/11, 100	13/13, 100	7/7, 100	5/5, 100
58	12/25, 48	4/7, 57.1	2/7, 28.6	6/11, 54.5	5/11, 45.5	5/9, 55.6	3/5, 60.
73	8/25, 32	2/7, 28.6	3/7, 42.9	4/11, 36.4	3/8, 37.6	5/9, 55.6	7/7, 100
88	24/25, 96	6/7, 85.7	7/7, 100	11/11, 100	7/8, 87.5	12/12, 100	5/7, 71.4
Total	165/225, 73.3	47/63, 74.6%	42/63, 66.7%	76/99, 76.8%	84/113, 73.7%	51/64, 79.7	37/48, 77.1%

P:Positive; T:Total,

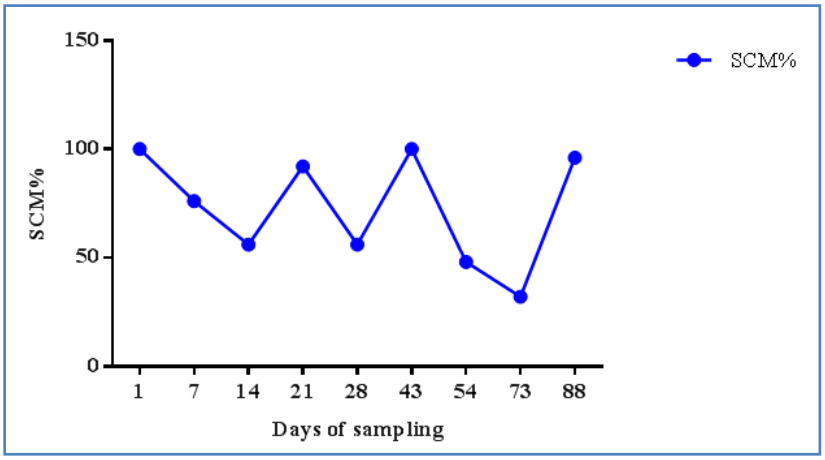

Figure 1 Prevalence of SCM based for period of 1st to 88th days.

For the purpose of studying the sequential prevalence of SCM, 25 cows were included in the study. The milk samples were collected from these cows at a weekly interval for five collections and followed by an interval of fifteen days collection for four times. The influence of factors such as the age, stage of lactation and milk yield were studied. A total of 225 milk samples were collected as detailed above.

2.2 Somatic Cell Counting (SCC) using nucleocounter

Fresh milk samples were used for SCC estimation using Nucleocounter (Chemo Metec, Denmark) following the instructions given by the manufacturer. Initially, five hundered microlitre of milk samples was mixed with equal quantity of the lysis buffer supplied by the manufacturer. The mixture was mixed gently to lyse the cells and was aspirated into the cassette by pressing the piston. The cassette was then inserted into the Nucleocounter and the SCC values were recorded. The SCC of > 5,00,000 cells / ml of test milk sample was considered cutoff to declare the positivity (Narayana & Iya,1954).

2.3 Multiplex polymerase chain reaction (mPCR)

Multiplex polymerase chain reaction was employed for the detection of bacterial pathogens in the milk samples. The specific primers *sip, pau A, 16S rRNA dys, alr* and *nuc* were used to detect *S. agalactiae, S. uberis, S. dysgalactiae, E. coli* and *S. aureus* respectively (Hegde, 2011) .

3 Results and Discussion

In the present study, a total of 225 collections of milk samples from cows were tested by SCC. A preliminary evaluation of these samples revealed 73.33% prevalence of SCM. Sample t-test was performed on SCC with different days of sampling. No significant difference was observed between any collections in SCC (P>0.05). The prevalence of SCM was at various time points during the study period is shown in Table 1 and Figure.1.

The average SCC observed in SCM milk samples by earlier workers has exhibited variation and this could be because of primary / secondary pathogens of udder. These pathogens affected the mean SCC values depending on degree of infection (Samanta et al., 2006). Various other factors such as cytoplasmic environment, calving season and persistent contact / exposure to dung, high environmental humidity can also influence the incidence of SCM along with increased SCC in milk samples (Madsen et al., 1992).

The SCC in milk from individual cows generally is a useful tool for monitoring the probability of intramammary infection, but must be complemented with bacteriological identification and enumeration. Bacterial culture is routinely used to diagnose mastitis, and culture results are often the basis for evaluating the quality and extent of a problem at the herd level. However, bacterial culturing of milk samples is laborious and time consuming. Polymerase chain reaction based detection of various pathogens in the milk is a rapid, sensitive and reliable method of detecting mastitis causing pathogens (Khan et al., 1998; Phuektes et al., 2001a; Phuektes et al., 2001 b; Phuektes et al., 2003; Shome et al., 2011; Shome et al., 2012).

In the present study, a total of 225 milk samples were screened for major bacterial pathogens and 113 organisms were revealed by mPCR. Of these 113, Maximum *S.aureus* (52.21%, 59/113) followed by *S.dysgalactiae* (15.93%, 18/113), *E.coli* (15.04%, 17/113), *S.agalactiae* (12.39%, 14/113) and *S.uberis* (4.43%, 5/113) were detected. This study was in accordance with Hedge et al., 2012 wherein mPCR results showed that *S.aureus* was a predominant pathogen detected (53.77%) followed by *S.dysgalactiae* (17.92%), *E.coli* (13.12%), *S.agalactiae* (11.32%) and *S.uberis* (3.77%) (Figure. 2).In this study, *S.aureus* was found to be the predominant pathogen prevailing at 52.21%. Sequentially, the days when SCC and mPCR (*S.aureus*) were positive for SCM, the milk yield showed a negative trend as per Radostitis et al. (2000).

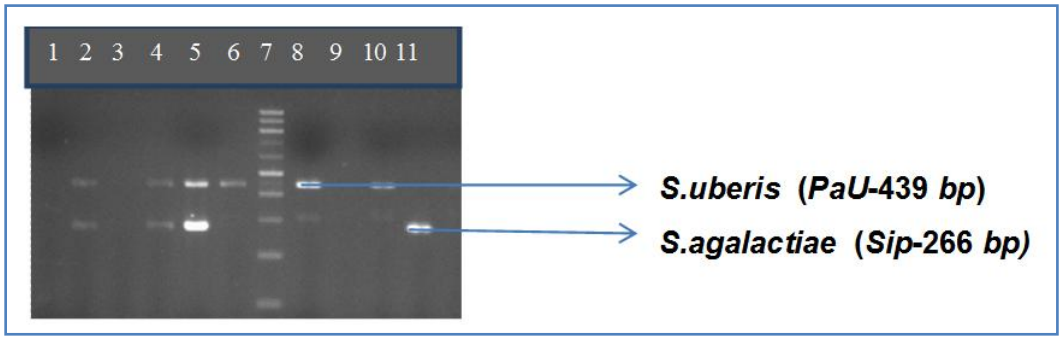

Figure 2 Two-tube Multiplex-Polymerase Chain Reaction (Tube 1) with milk samples for *S.uberis* (*PaU*-439 *bp*) and *S.agalactiae* (*Sip*-266 *bp*). [**Lane Details** : Lane 1,3,9: Negative for *S.uberis* and *S.agalactiae;* Lane 2,4,5: Positive for *S.uberis* and *S.agalactiae;* Lane 6,8,10: Positive for *S.uberis;* Lane 7: Ladder (100bp); Lane 11: Positive for *S.agalactiae]*

Only on certain days (14, 28, 54 and 73 days), there was not much influence on milk production though the organisms detected. As a predominant mastitis causing pathogen, *S.aureus* is able to survive for longer time on skin (McDonalds, 1977) and inside the neutrophils of the mammary gland (Craven & Anderson, 1979; Sandholm et al., 1990) thus protecting itself from the action of antibiotics. This may be the reason that the SCC level remained elevated in our study on day 21st and 43rd due to intra cellular localization and in turn being protected from being acted upon by the antibiotics and acting as a immunogen. Boulanger et al.(2003) postulated that basal NF-kB activity is required for penetration of *S. aureus* into mammary epithelial cells, and that pharmacological NF-kB inhibitors could be used to reduce the intracellular infection of *S. aureus* (Hogan & Smith, 2003).

Yet another predominant mastitis causing pathogen detected is Streptococci. With respect to the prevalence of environmental streptococcal mastitis, a large proportion of variability in its incidence and, both between geographical locations and within a single herd, can be ascribed to a number of independent variables such as season of the year, stage of lactation, parity, and various management practices (Hogan et al., 1989; Pankey et al., 1996; Hogan & Smith, 2003). *Streptococcus agalactiae* is one of the obligate pathogen of mammary gland in case of bovines, colonizing the teat canal (Dodd, 1983). In our study, *S.agalactiae* was prevailing at 12.39%.

The persistence of this organism is attributed to ill hygiene and general managemental factors. Further, *S dysgalactiae* and *S uberis* are other species associated with SCM. However, these species are not an obligate pathogen of mammary gland and they enter the udder by injurying the teat (Cullor & Tyler, 1996). Furthermore, Sandholm et al. (1990) reported that *S.dysgalactiae* is a predominant pathogen associated with summer mastitis and it's frequent isolationfrom heifers and dry cows. While *S.uberis* being opportunistic could thrive and proliferate in tissues other than mammary glad. including lips, haircoat, tonsils and the rectum of cows (Bramley et al., 1979). Todhunter et al. (1994) opined that the rate of S*treptococcal* infection was high in summer season in lactating and dry cows. Further, the type of housing and associated managemental practices in the dairy farm could lead to the contamination of bedding materials and inturn expose teats to streptococci in the environment (Smith & Hogan, 1993; Smith & Hogan,1995). However, these factors do not appear to have contributed in the present study as the farm under investigation was well organized.

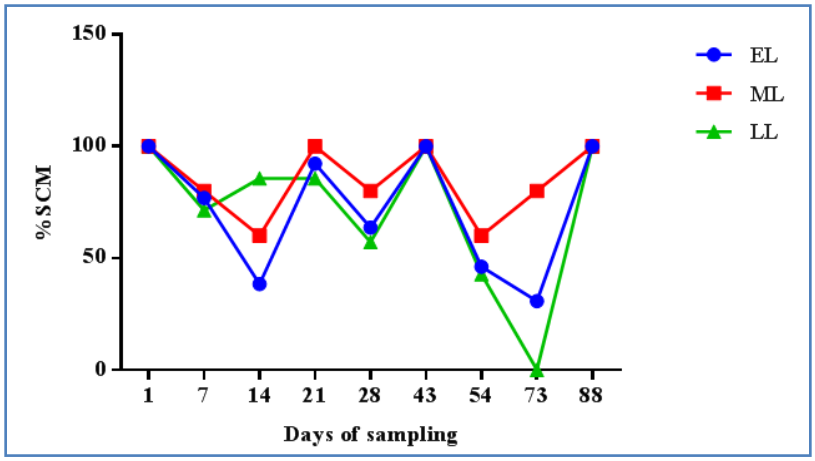

Figure 3a Prevalence of SCM based on SCC in different stage of lactation.

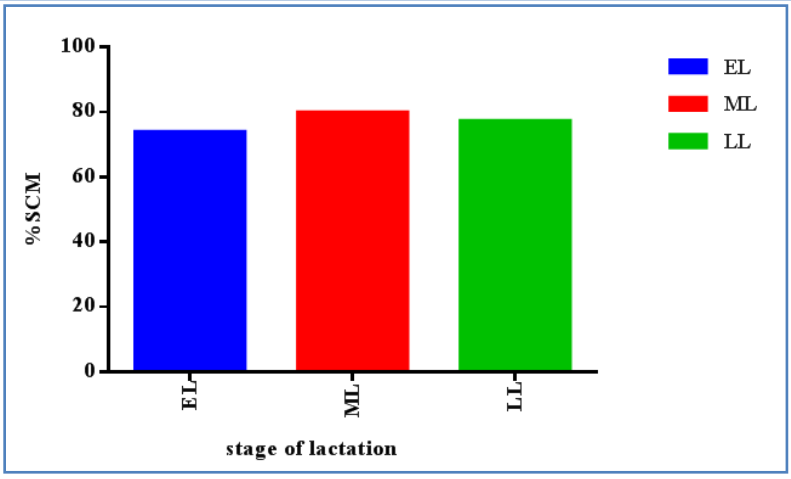

Figure 3b Percentage prevalence of SCM based on SCC in different stage of lactation groups.

Escherichia coli is a Gram negative organism reported from the most of the bovine mastitis cases in both clinical mastitis (CM) and SCM. Although the infections due to *E.coli* are of short duration of <28 days (Todhunter et al., 1991), Many researchers reported the recurrent coliform mastitis and persistent infections due to *E.coli* in dairy animals. These studies concluded that the severity of mastitis due to *E.coli* is mainly related to host factors (Hill et al., 1979; Bradley & Green, 2001). In present study, *E.coli* was found prevalent at 15.04%, the days when SCC and mPCR were positive for SCM with the milk yield showing a negative trend. However, on certain days (28th, 54th and 73rd), there was no change in milk yield even in the presence of organism and this could be attributed to persistence stage / latent infection / carrier stage of infection / self cure, which is in agreement with Jayarao et al. (1999) who also reported that prevalence of IMI due to environmental pathogens might increase in the absence of contagious pathogens. Similarly, Schukken et al. (1989) also opined that low count of SCC due to decreased prevalence of contagious pathogens might lead to high prevalence of IMI due to environmental pathogens.

The appearance of mastitis pathogens in milk samples from a random sample of the cow population of this study revealed relationships between microbiological diagnosis and milk yield similar to those previously reported from clinical IMI. Multiplex PCR showed a similar variability as reported earlier (Hegde, 2011) and the benefits we experienced with mPCR were rapid, simple and accurate in revealing organisms. Viewed as a whole, study indicates that a positive diagnosis of *S. aureus* and *Streptococcus* species according to microbiological milk analysis of clinically normal cows correlates with production potential as opined by Reksen et al. (2007). In this study, M-PCR employed was a qualitative approach which detected the predominant species of bacteria involved in the SCM cases but not quantitative. It is necessary to estimate the bacterial load of different / various pathogens associated with SCM in order to understand the influence of the load of etiological agents on occurrence of SCM.

In the present study, the prevalence of SCM based on SCC during EL, ML and LL was 73.68%, 79.68% and 77.1% respectively (Figure.3a and 3b). The one-way ANOVA was performed on SCC of samples from the first collections to day 88, at different stage of lactation. No significant differences was observed between any stage of lactation with respect to prevalence of SCM (P>0.05). In the present study, the prevalence of SCM based on SCC was high in the third lactation (75%) which is in agreement with Islam et al. (2011) and Sripad et al. (2013) who have also reported high prevalence of SCM (47.05% and 68.89%) during the third lactation. It is well established fact that bovine immune system is less capable of battling pathogens during the periparturient period. Although exact causes for a compromised immune system are not fully understood, they are believed to be at least influenced by hormonal and metabolic changes associated with pregnancy, parturition, and onset of lactation (Burvenich et al., 2003), Although exact causes for a compromised immune system are not fully understood, they are believed to be at least influenced by hormonal and metabolic changes associated with pregnancy, parturition, and onset of lactation (Burvenich et al., 2003), Additionally, during the peripartum period a substantial reduction in the levels of trace elements, protein and energy in blood that may result in occurrence of disease (Burvenich et al., 2003). Both CM and high milk production occur more commonly in older cows and in cows early in lactation (Bartlett et al., 1990).

The present study did not reveal any association between the stage of lactation and the prevalence of pathogens. The m PCR revealed the prevalence of predominant pathogens at 35.96% (41/113) in EL; 29.68% (19/64) in ML and 33.33% (16/48) in LL Further, the application of m-PCR revealed 31.86% *S.aureus* (36/113) , 1.77% *S.agalactiae* (02/113), 8.85% *S. dysgalactiae* (10/113), 0.89% *S.uberis* (1/113), 8.85% *E.coli* (9/113) in EL; 8.85% *S.aureus* (10/113), 6.2% *S.agalactiae* (7/113), 1.77% *S.dysgalactiae* (2/113), 1.77% *S.uberis* (02/113), 1.77% *E.coli* (2/113) in ML and 11.5% *S.aureus* (13/113), 1.77% *S.uberis* (02/113), 4.43% *S.agalactiae*

(5/113), 4.43% *S.dysgalactiae* (5/113) and 4.43% *E.coli* (5/113) in LL . In all the stages of lactation, *S.aureus* was found predominant. Among three stages of lactation, maximum number of milk samples (50%, 58/113) revealing the presence of pathogens were obtained from cows in the EL. However, this did not result in reduction in the milk yield during the EL. Based on this observation, it is evident that the varied distribution of organisms in different stages of lactation did not influence the prevalence of SCM and in turn affected the milk yield.

Singh & Ludri (2001) opined that the milk yield varied significantly ($p<0.01$) during different stages of lactation and was also negatively correlated with SCC, whereas in our study there was low correlation between milk yield and SCC ($r = 0.038$). Such weak correlation observed in the present work may be due to varied number of cows in different lactation stages. Furthermore, in our study, at different stages of lactation, the milk yield during the EL was at highest 3184.8 liters/cow (35.32%), with milk loss of 242.2 liters/cow (2.82%), it was followed by ML with milk production at 2074 liters/cow (22.99%) and milk loss of 792 litres / cow (8.78%). Where as in LL, the milk production was 1609.3 litres / cow (17.85%) with milk loss of 1113 litres / cow (12.34%). The milk loss was highest in LL (12.34%), followed by ML (8.78%) and least loss in EL (2.82%). The high milk yield in the EL than ML and LL in the present study is in accordance with the previous reports. Further, none of the tests employed indicated high prevalence of SCM in the EL as compared to ML and LL. Overall, the findings of the present study indicated that the stage of lactation did not influence on the prevalence of SCM.

In the present study, the animals were grouped into three age groups namely 3-5 years, 5-7 years and 7-11 years and the prevalence of SCM in these three age groups is 73.33%, 66.66% and 76.76% respectively (Figure 4a & 4b). Furthermore, the one-way ANOVA was performed on SCC with different age groups for sampling of all 9 collections. Although, no significant difference was observed between the age groups with respect to SCC ($P>0.05$), and in turn the prevalence of SCM, relatively, the prevalence of SCM was higher in the age group 7-11 years. The SCC revealed the high prevalence of SCM (76.76 %) in 7-11 years as compared to 3-5 and 5-7 years age groups. The high prevalence of SCM with advancing age and in older cows draws support from the findings of earlier workers (Radostits et al., 2000; Qadri et al., 2005; Ul-Hah & Malik, 2009).

Rahman et al. (2009) also reported that the prevalence of SCM significantly increased with age in dry as well as in wet season. Islam et al. (2011) reported that the prevalence of SCM was significantly higher in the age group of animals more than 13 years at 47.61 percent. The high prevalence of SCM in older cows could be attributed to suboptimal host defensive mechanisms (Dulin et al.,1988), prior exposure to the pathogens, cumulative SCM and carrier stage (Akbar et al., 2004). The higher prevalence of SCM in older animals than in younger cows could be attributed to suboptimal defense mechanism as indicated by Dulin et al. (1988). In addition, might be the other reason for the observation of as opined by Workineh et al. (2002). The higher prevalence of SCM in the aged cross bred cows as observed in the present study was also in accordance with Samanta et al. (2006) and Mustafa et al. (2007) .

The present findings are in agreement with the general observation that the mastitis incidence and SCC levels are both higher in older cows. This paradoxical finding could be well related to the functionality of the resident milk cells where in milk PMN in primiparous cows have been found to have a higher viability and ROS production as compared to older animals (Burvenich et al.,2003; Samanta et al., 2006). Further, the findings of the present study are also supported by the observation of Hogan & Smith (2003) that the rate of IMI during the dry period was greater in multiparous cows compared with primiparous cows.

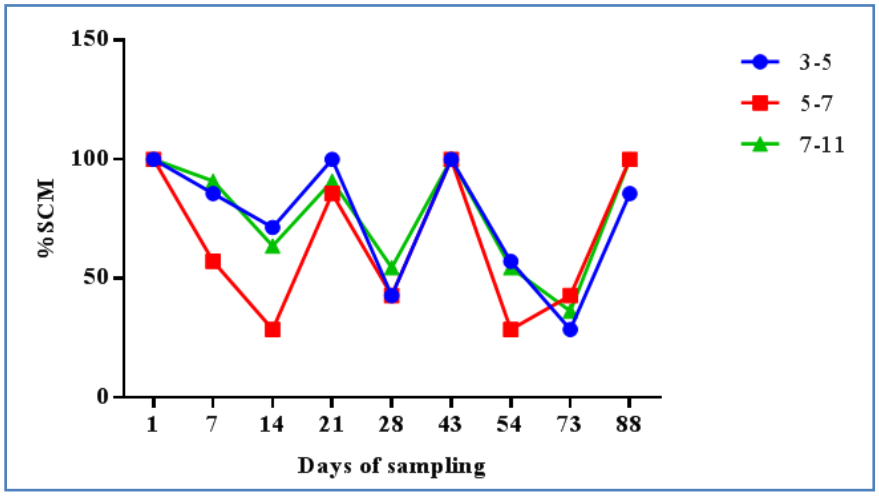

Figure 4a Prevalence of SCM based on SCC in different age groups.

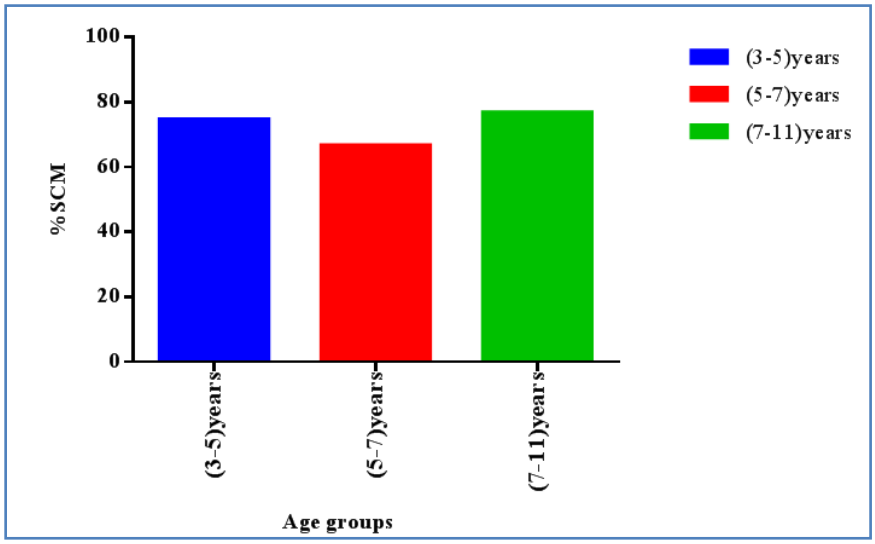

Figure 4b Percentage prevalence of SCM based on SCC in different age groups.

High production cows appear to be at higher risk of developing CM. Also, both CM and high milk production occur more commonly in older cows Bartlett et al. (1990). Further, high prevalence of SCM in aged cows could also be attributed to the dilatation or partial opening of teat canal in case of older cows due to repeated milking. This encourages the introduction of environment and skin–associated microorganisms into the teat canal, leading to SCM and milk production losses. In addition, cows that are multiparous / aged have poor defence mechanism. Furthermore, elevated SCC due to minor pathogens could protect the mammary gland from major pathogens (Burvenich et al., 2003). Nevertheless, the correlation between SCC and the immune response of the udder to infection is complex and unclear.

With respect to the detection of major mastitis causing bacterial pathogens in different age groups of cows, the application of mPCR revealed the prevalence of *S.aureus* at 17.77 % (20/113), *S. dysgalactiae* at 2.66 % (3/113), *E.coli* at 4.43% (5/113), *S.agalactiae* at 1.77 % (02/113), *S.uberis* at 1.77 % (2/113) in age group of 3-5 years; *S.aureus* at 11.5 % (13/113), *S.agalactiae* at 3.54 % (4/113), *S.dysgalactiae* at 5.31 % (6/113), *E.coli* at 4.43 % (5/113) in age group of 5-7 years and *S.aureus* at 23.0% (26/113), *S.agalactiae* at 7.1 % (8/113), *S.dysgalactiae* at 7.1 % (8/113), *E.coli* at 5.31 % (6/113) and *S.uberis* at 3.54 % (4/113) in age group 7-11 years (Figure.4a and 4b). In all the age groups of cows, *S.aureus* was found predominant. Among three age groups, maximum number of milk samples (46 %, 52/113) revealing the presence of pathogens were obtained from cows in the age group 7-11 years. However, this did not affect the milk yield during the LL. In the study of Bartlett et al. (1990), pluriparous cows showed a milk loss of 2.06 times compared to that in the first lactation cows, milk loss of 1.40 times was observed in mastitic cows prior to 150 days in lactation compared to other cows and a milk loss of 1.37 times was seen in cows with mastitis during winter compared to summer season. However, in this study, the identity of the mastitis causing agent isolated from the clinical case was not strongly associated with the drop in milk production in the 60 day following clinical onset in Based on the observations of the present study and Bartlett et al. (1990), it is evident that the varied distribution of organisms in different age groups did not influence on the milk yield.

Conclusion

In conclusion, the present study revealed the high prevalence of variable SCM pattern in milking cows by SCC (73.33 %) using sequentially collected milk samples over a period of 88 days in an organized farm. No specific pattern of prevalence of SCM was observed in the sequentially collected milk samples during the study period. The prevalence of SCM was not influenced by the stage of lactation. In all the stages of lactation, *S. aureus*, *Streptococci* and *E.coli* were detected with the predominance of *S. aureus*. The varied distribution of organisms in different stages of lactation did not influence the prevalence of SCM. Further, the high prevalence of SCM was noticed in aged cows. The M-PCR revealed the presence of *S. aureus*, *Streptococci* and *E.coli* with the predominance of *S. aureus* in all the milk samples collected from all the three age groups. Among these groups, maximum number of milk samples (46 %, 52/113) revealing the presence of pathogens were obtained from cows in the age group 7-11 years. The M-PCR assay employed in the present study was an easy and rapid method to detect the predominant pathogens causing SCM. Hence, the regular analysis of milk samples by M-PCR may be a useful tool for determining the herd status with regard to the detection of contagious and environmental mastitis pathogens. The result indicated the presence of both contagious and environmental mastitis pathogens. This emphasizes continuing need to concentrate on control both contagious pathogen such as *S. aureus* and environmental pathogen especially *E.coli* through sequential monitoring of

SCM through application of SCC, M-PCR and proper managemental practices.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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DIAGNOSIS OF ANIMAL RABIES: COMPARISON OF DIRECT FLUORESCENT ANTIBODY TEST (dFAT), REVERSE TRANSCRIPTASE -PCR AND REAL-TIME PCR

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KEYWORDS

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ABSTRACT

Rabies is endemic in most parts of India, with the exception of Andaman and Nicobar, Lakshadweep islands and to some extent Nagaland. For prevention and control it is essential to rapidly and precisely diagnose rabies. In this study, we used three diagnostic methods, direct fluorescent antibody test (dFAT), reverse transcriptase polymerase chain reaction (RT-PCR) and real time reverse transcriptase polymerase chain reaction (RT-qPCR) to detect the rabies virus in suspected animal brains. Out of the 80 animal brain samples tested, 64 (80%) were positive for rabies according to the RT-qPCR. Compared to the RT-qPCR, the sensitivities of dFAT and RT-PCR were 95.31% and 96.88%, respectively. The specificities of dFAT and RT-PCR were on far with qRT-PCR. Even though the dFAT findings did not completely coincide with results obtained from RT-PCR and RT-qPCR, dFAT appears to be a fast and reliable assay that can be used to analyze fresh brain samples. But in countries like India where temperature reaches 50°C during summer and lack of diagnostic facilities and trained personnel to carry out the dFAT at field level, the suspected samples will be usually sent to National/Regional Disease Diagnostic Laboratory /State veterinary or agricultural universities for rabies diagnosis. In summary the molecular methods RT-PCR and RT-qPCR can serve as quick and rapid diagnostic methods for animal rabies in India.

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1 Introduction

Rabies is fatal non-suppurative encephalitis caused by RNA virus of genus *Lyssavirus* in the family Rhabdoviridae. In India the disease is enzootic in nature with dogs as the main vector of transmission accounting for 20,000 human deaths per year (Sudarshan et al., 2007). The detection of negribodies by sellar's staining is an old method for diagnosis. The development of direct fluorescent antibody technique (dFAT) which according to WHO, is a gold standard for rabies diagnosis because of short duration, low cost and higher sensitivity. As an adjunct to dFAT mouse inoculation is also carried out especially in developing countries, which is also highly sensitive method but requires several days to get the result (Chhabra et al., 2005).

In tropical countries like India, where the sample shipment may take longer time to central laboratories for diagnosis with high probability of break in cold chain leading to decomposition, In such condition the reverse transcriptase polymerase chain reaction (RT-PCR) and real time reverse transcriptase polymerase chain reaction (RT-qPCR) which have high sensitivity may serve as better diagnostic assays. Many studies carried out to develop and validate RT-PCR and RT-qPCR for diagnosis of rabies in human (Hughes et al., 2004; Nagaraj et al., 2006; Wacharapluesadee et al., 2008; Nadin-Davis et al., 2009). Whereas, few independent single test based studies are carried out on animal rabies diagnosis especially in Indian sub content (Gupta et al., 2001; Jayakumar et al., 2003; Praveen et al., 2007; Kaw et al., 2011). The present study describes the comparative evaluation of dFAT, RT-PCR with syber green Real time PCR for the diagnosis of rabies in domestic and wild animals in India.

2 Materials and Methods

2.1 Study samples

A total of eighty rabies suspected samples collected from different species and different geographical regions (Andhra Pradesh:3, Gujarat:6, J&K:1, Karnataka:11, Kerala:10, Maharashtra: 8, Orisa:2, Punjab:8, Rajasthan:12, Tamil Nadu: 4, Uttarakhand:3 and Uttar Pradesh:12) of country were used in the study.

2.2 Direct fluorescent antibody technique (d FAT)

The FAT was performed according to the procedure described by the Office International des Épizooties (OIE) and World Health Organization (WHO). Briefly, the impression smears were prepared from different portions of brain and were fixed in chilled acetone for 2 hrs. The slides were encircled around the smear and were immersed in PBS (pH 7.2) for 5 min. They were incubated with FITC conjugate anti-rabies antibody (BioRad, USA) for 30 min in humidified dark chamber at 37°C. The slides were washed with PBS thrice in slide holding glass trough by creating current with magnetic stirrer. After washing, slides were mounted examined under fluorescent microscope at 400 nm (Nikon, Japan). The presence of dusty apple green fluoresce was taken as positive signal. Brain samples from mice infected with challenge virus standard (CVS) and normal mouse brain were as positive and negative controls, respectively.

2.3 Isolation of RNA

Total RNA from brain tissue was extracted by TRIZOL method. In Brief, 50-100 mg of brain tissue was homogenized in 1 ml of Trizol-LS reagent (Invitrogen, USA). After incubation, 200 µl of chloroform (Sigma, USA) was added and mixed vigorously for 15 seconds and incubated for 10 min on ice. The aqueous phase collected after centrifugation @ 12000 rpm for 10 min at 4 ℃ was mixed with 900 µl of isopropanol, followed by centrifugation at 12000 rpm for 10 min at 4 ℃. The RNA pellet was washed with 1 ml of 70% ethanol, dried and dissolved in 50 µl of nuclease free water and concentration of RNA was estimated by spectrophotometer (NanoDrop ND-1000, USA).

2.4 cDNA preparation

The cDNA synthesis was carried out using Reverse Transcription System (Promega, USA) as per the recommended protocol. Briefly, 2 µl of total RNA (~ 1.0 µg) was incubated for 10 min at 70℃ and quickly chilled on ice and centrifuged briefly. Then final reaction volume of 20 µl was prepared by adding 4.0 µl of MgCl2 (25 mM), 2.0 µl of RT 10X buffer, 2.0 µl of dNTP mixture, (10 mM), 0.5 µl of RNasin, 0.6 µl of AMV Reverse Transcriptase (20 IU/µl), 1.0 µl of Random Primers (0.5mg/ml), and 7.9 µl of Nuclease free water. The reaction mixture was incubated at room temperature for 10 min followed by 42 °C for 60 min. Reverse Transcriptase enzyme was inactivated at 95℃ for 5 min with final incubation at 4^{o}C for 5 min.

2.5 Reverse transcriptase Polymerase chain reaction (RT-PCR)

RT-PCR for detection of rabies virus targeting most conserved nucleoprotein gene specific two sets of primers (Table 1) designed based on sequences available from our previous study was carried out (Reddy et al., 2011). Briefly, 25 µl reaction mixture (2.5x master mix- 10 µl, $MgCl_2$,10 mM- 1.0 µl, Primer Forward- 0.5 µl, Primer Reverse-0.5 µl, Template cDNA-1 µl and Nuclease free water- 12 µl) using 2.5x master mix (5 PRIME, USA) was prepared. The amplification was carried out in thermocycler (Eppendorf, Germany) with an initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C/1 min, 53^{o}C/1 min and 72^{o}C/1 min, and a final extension step of 72^{o}C for 10 min. the amplified products were electrophoresed on 1% agarose and were photographed.

Table 1 Oligonucleotide primers used for RT-PCR and RT-qPCR to diagnose rabies.

Primer	Nucleotide sequences (5´-3´)	Nucleotide position*	Sense	Rabies gene	Size of amplicon (bp)
RABNF1	GATTGAGCATCTATATTCAGC	648-688	+	N	200
RABNR2	GAGGAACGGCGGTCTCCTG	888-869	_		
RABNF2	ACTGATGTAGAAGGGAATTG	340-359	+	N	533
RABNR2	GAACGGAAGTGGATGAAATA	872-853	_		

2.6 Real time PCR (RT-qPCR)

Syber green RT-qPCR was performed in 20µl reaction volume comprised of 8.0 µl of nuclease-free water, 0.5 µl each of the primers (table.1), 10 µl of 2x syber green master mix (Finnzymes, Finland) and 1 µl of cDNA. The reactions were carried out in thin wall clear PCR strip tubes with clear strip caps (Axygen, USA) in an MX3000P quantitative PCR system (Stratagene, USA). The reaction was performed for 40 cycles with 55°C for 30 seconds. Positive control (cDNA from mouse brain infected with CVS) and negative control (cDNA from PBS control mouse brain) assays were run along with NTC (no-template control).

2.7 Calculation of sensitivity and specificity

Sensitivity was calculated with the formula [TP/(TP+FN)] × 100 where TP was the number of samples with true-positive results as determined by the reference assay (qRT-PCR) and FN was the number of samples with false-negative results. Specificity was defined as [TN/(TN+FP)] × 100 where TN was the number of samples with true-negative results and FP was the number of samples with false- positive results.

3 Results

In the present study comparison of three diagnostic methods (dFAT, RT-PCR and RT-qPCR) was carried out with a total of 80 suspected rabies brain samples from wide host range (beer; 2, buffalo;8, cattle;15, dogs; 28, horse; 5, human; 6, hyena; 5, goat; 8 and jackal; 3) and from different geographical regions of the country were used in the study: Uttar Pradesh-12, Uttarakhand-3, J&K-1, Punjab-8, Rajasthan-12, Gujarat- 6, Maharashtra- 8, Karnataka-11, Kerala-10, Tamil Nadu-4 and Orisa-2 Andhra Pradesh 3. When these 80 animal and human brain samples were tested; 61 were positive according to the FAT (Figure. 1A & Figure.1 B), 62 were positive by RT-PCR and 64 were positive by RT-qPCR. All the three assays gave negative for remaining 16 brain samples. Sensitivities of dFAT and RT-PCR were 95.31 and 96.88% respectively and specificities of dFAT and RT-PCR were on far with RT-qPCR (Table. 2).

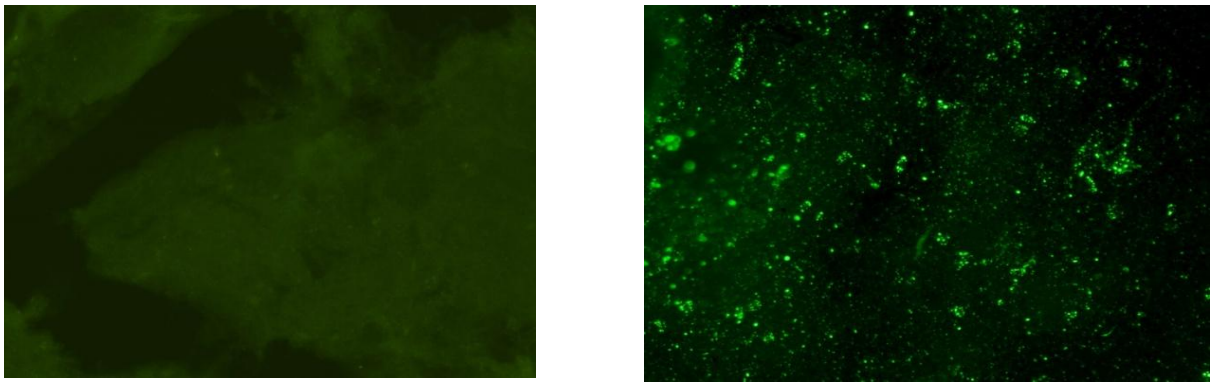
Conventional RT-PCR using a primer set that amplified the N gene of rabies virus was able to detect viral RNA in 62 samples with both sets of primers covering short (RABNF1 and RABNR1) as well as high length of genome (RABNF2 and RABNR2). The band intensity of the amplified gene varied among the samples (Figure. 2). However, RT-qPCR could detect the N gene in 64 samples without any non-specific reactions with specific melting temperature (82.24-83.11^{0}C).

4 Discussions

Rabies is endemic in most parts of India, with the exception of Andaman and Nicobar, Lakshadweep islands and to some extent Nagaland. National survey by the Association of the Prevention and Control of Rabies in India estimated a total of 20,000 human deaths due to dog bite each year (Sudarshan et al., 2007). Although the loss of livestock due to rabies is significant, there are only few publications on estimates of the incidence of rabies in livestock (Knobel et al., 2005). In India, dogs play an important role as the reservoir and transmit rabies to humans and domestic animals in urban cycle, while jackals, wolves and foxes maintain the rabies virus in sylvatic cycle (Reddy et al., 2011). Monitoring and surveillance of any disease requires rapid diagnostic tests. In the present study we first screened the all suspected rabies brain by dFAT as it is considered as the gold standard for rabies diagnosis by WHO, but in our study with dFAT we were able to detect only 61 out of 64 cases which found positive by RT-qPCR (Table. 2).

Table 2 Sensitivity and specificity of dFAT, RT-PCR, and qRT-PCR for diagnosis of rabies virus in the field brain samples.

		qRT-PCR			
		P	N	Sensitivity	Specificity
dFAT	P	61	0	95.31	100
	N	3	16		
RT-PCR	P	62	0	96.88	100
	N	2	16		



(A) (B)

Figure 1 Brain impression smears showing no specific (a) and specific (b) rabies fluorescence signals

The low sensitivity of dFAT might be attributed to improper storage of samples, condition of sample, load of virus, stage of disease and preservative (Trimarchi & Smith, 2002; Wacharapluesadee & Hemachudha, 2010). The decomposed samples leading to loss of antigenic proteins may lead to false negative diagnosis by dFAT this can lead to problem in tropical and subtropical countries where transportation of specimens to a regional diagnostic laboratory is often delayed (Loza-Rubio et al., 2005). The other disadvantage is confirmatory assays for dFAT comprise the rabies virus isolation and mouse inoculation test (MIT) but these will also require days to weeks until final diagnosis, so these disadvantages and the identification of new strains of the virus encourage the use of new techniques like RT-PCR and RT-qPCR that are rapid, sensitive, specific and economical for the detection and research of the Rabies Virus are being increasingly used in diagnosis and research laboratories (Silva et al., 2013).

Out of 64 cases, 62 cases were found positive with rabies virus 'N' gene specific primers with specific amplification with 96.88% sensitivity by RT-PCR. The higher sensitivity and specificity as compared to dFAT has also been reported earlier and might be due to decomposition of tissue during which the RNases degrade the genome into smaller segments (Smith et al., 2000; David et al., 2002). Rojas et al. (2006) reported successful detection of rabies virus genome up to 23 days in the brain samples stored at ambient temperature. The low sensitivity in the present study might also be attributed to lower number of nucleic acid copies leading which inturn might have lead to negative by RT-PCR and positive by RT-qPCR. The RT-PCR besides many other advantages like earlier detection of rabies viral infection compared to DIF and Seller staining can also be useful in strain identification with sequencing for molecular epidemiology (Biswal et al., 2012).

Nowadays RT-qPCR based diagnostic assays are more favored than conventional reverse-transcription PCR methods by several laboratories, because RT-PCRs will generates the risk of post amplification processing, cross-contamination, does not allow an exact quantification of genome copies and does not include tests for specificity (Belak & Thoren, 2001; Wacharapluesadee & Hemachudha, 2010). With nucleic acid detection techniques fragmented genome can be detected depending on the size and the primers covering that length of genome as in the case of real time PCR, where we could diagnose 64 samples as positive. In the present study the more number of samples were found rabies positive by RT-qPCR compared to RT-PCR even though the length of genome covered is same (200bp) this might be due to amount of viral genome in the sample also determines the sensitivity of these two techniques.

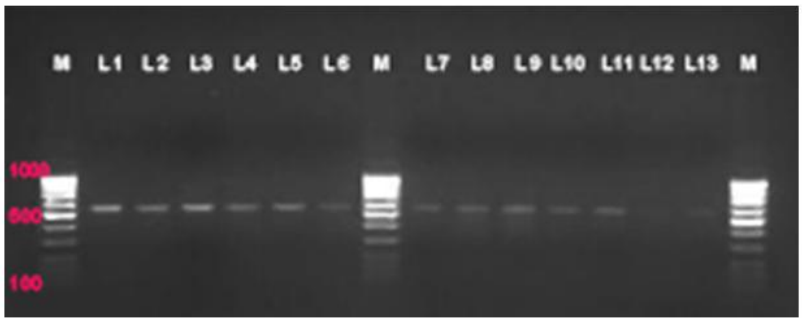


Figure 2 Gel photo showing amplified N (533bp) gene of rabies virus from different field rabies isolates. M - 100bp marker, L1– Positive control (CVS) and L2-L13 are field samples.

Hughes et al. (2004) reported single mutations for the North American RABV strains in the region of the primers or the probe can alter the sensitivity of the PCR. The above observations were eliminated in the present study by designing primers after sequencing more than 40 isolates. But more studies are warranted targeting different portions of rabies genome as the genetic diversity among rabies viruses may hamper the use of a single assay and rabies surveillance may benefit more from the use of a pan-lyssavirus primer SYBR green assay rather than a strain or specific based assay (Fooks et al., 2009).

Conclusion

The results of the present study demonstrated the high potential of RT-qPCR over RT-PCR and dFAT for the diagnosis of rabies in domestic and wild animals in India.

Conflict of interest

All Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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DYSTOCIA DUE TO FETAL MALDISPOSITION AND MALPRESENTATION IN A NAGALAND MITHUN (*Bos frontalis*)

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KEYWORDS

Mithun

Maldisposition and malpresentation

Caesarean section

ABSTRACT

This short communication of a case report of dystocia in a pluriparous Nagaland mithun strain was due to fetal maldisposition and malpresentation. Severe left lateral deviation of head and neck and dorso-pubic-anterior presentation of the fetus caused the maldisposition in Nagaland mithun strain. It was successfully removed with caesarean section and the animal recovered uneventfully.

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1 Introduction

The mithun is a unique bovine found in some of the North Eastern states and are four particular strains in Indian sub-continent namely Nagaland, Arunachal, Mizoram and Manipur strain. A small number of mithun is reported in neighbouring countries such as Bhutan, Myanmar, Yunan province of China and Bangladesh and believed to be a descendent of wild gaur (Simoons, 1984). In general, mithun is reared in free-range system as the traditional practice and the mithun farmers/owners rear their mithun in the village community jungles and forest in group or herd. In general, no reproductive problem was reported in the free ranging mithun rearing forest areas, but some reports were observed in the semi-intensive mithun farm (Perumal et al., 2012; Perumal et al., 2013a; Perumal et al., 2013b). Deviation of the neck and head are very common group of abnormal posture in anterior presentation resulting difficult in birth in different livestock animal species (Roberts, 1971).

The present abnormal deviation may be observed and corrected in any directions and lateral deviation of the neck and head is reported very commonly in unipara and the prognosis of these types case is very serious especially when the deviations are due to muscle contractures and the fetus is dead in bovine species (Sane et al., 1994). The reproductive problem such as dystocia is very rare in this bovine species when compared to other bovine and bubaline species and moreover no such report was observed regarding the dystocia in this precious species and its successful management using caesarean section. Therefore the present case report keeps on record a case of dystocia due to lateral deviation of head and neck and dorso-pubic-anterior presentation and its management in Nagaland mithun strain.

2 History and clinical examination

A Nagaland mithun of age 6 year was attended at Porba village, Phek District, Nagaland with a history of reduced feed intake, discomfort and a soiled white cord hanging from vagina. The respiration, temperature and pulse were slightly increased. Drooling of saliva from mouth and dry muzzle and congested conjunctival mucus membranes were observed in the present case. Birth canal was dry and oedematous, a fully dilated cervix with hard in consistency and the foul smelling foetal fluids were observed through per-vaginal examination.

The foetus was in dorso pubic position with anterior longitudinal presentation with severe lateral deviation of neck and head and the dead fetus was diagnosed. Finally the case was diagnosed as severe fetal dystocia because of dorso-pubic-anterior presentation and severe left lateral deviation of neck and head based on per rectal and per vaginal examination. Fertile and hard attempts were done to relieve the fetus using mutational operation, but no fruitful result. Therefore the caesarean section was performed to relieve the fetus.

3 Treatments and Discussion

Epidural anaesthesia (2% Lignocaine HCl; 7 ml) was given followed by the animal was properly restrained in right lateral recumbency and left lower flank was prepared for caesarean section. 2% lignocaine HCl was for local infiltration and caesarean section completed as per prescribed surgical protocol. A male dead emphysematous fetus was removed with affected fetal membranes from the uterus in the section. Surgical site was properly closed and dressed with care as per standard surgical procedure. General post operative care was provided and the uneventfully animal was recovered.

In this case report, the fetal mal-disposition and mal-presentation resulted dystocia, improper and lack of timely intervention leads to fetal death and finally emphysema. Johanson & Berger (2003) reported 49% of perinatal mortality was related with unassisted births/delivery. Following death of fetus within 1-3 days, there was invasion of microorganisms from the lower reproductive tract (vagina) resulted dead foetuses with emphysema (Purohit & Mehta, 2006). Similarly, in the present case report, the fetus was emphysematous, uterine wall contracted around the fetus, the vaginal canal was dry and the vaginal discharge was foetid and watery.

All these observation indicated that death of foetal would have occurred from 1 to 3 days prior to the surgical caesarean section. The fetal mal-dispositions were associated with emphysema of the foetus, deep incision on the foetus to release the gas and forced traction and partial fetotomy can relieve dystocia as in the present study (Purohit et al., 2012; Perumal et al., 2013c). In the present dystocia case report, caesarean section was performed because other fertile attempts failed to relieve/correct the dystocia and further the health of the dam was determined as fair to withstand caesarean section.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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HISTOPATHOLOGICAL FEATURES OF CUTANEOUS TUMOURS ARISING FROM THE VASCULAR ENDOTHELIAL CELLS IN DOGS

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KEYWORDS

Cutaneous tumours

Dogs

Hemagioma

Hemangiosarcoma

Lipohemangioma

ABSTRACT

Cutaneous growths arising from the endothelial cells of blood vessels were studied in three dogs (Two in male cocker spaniels and one in male Labrador). All the three growths were surgically excised and referred to the Department of Veterinary Pathology, Tirupati for histopathological confirmation during the period from August 2015 to December 2015.Gross examination revealed, firm reddish, soft dark red and soft greyish red coloured masses and are diagnosed based on histopathological features as hemangiosarcoma, cavernous hemangioma and lipohemangioma respectively. In the case of hemangiosarcoma, severe necrosis along with the presence of blastomyces organisms as a secondary infection was also noticed. Occurring of these tumours might be because of exposure of lightly pigmented areas to Ultra Violet radiation.

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1 Introduction

In canines, skin, soft tissue and mammary glands are the commonest sites for the development of various benign tumours compared to malignant (Anudep et al., 2003; Aleksic-Kovacevic et al., 2005; Murphy, 2006). Based on the survey on dermatological conditions of dogs conducted by Hill et al. (2006) in UK, cutaneous parasitic infestations, bacterial infections and neoplasia were noticed in majority of the cases. Of all the cutaneous neoplasms of dogs, tumours of vascular origin and transmissible venereal tumours were considered as more common one (Chikweto et al., 2011).

The etiological factors that are responsible for the occurrence of these tumours were not well understood. Some authors reported that, breed and ultraviolet radiation are the major risk factors for various canine cutaneous hemangiomas, hemangiosarcomas and squamous cell carcinomas (Hargis et al., 1992; Nikula et al., 1992; Chikweto et al., 2011). Spontaneous tumours of blood vessel endothelial cells were described commonly in the dog, less frequently in the cat and horse, and sporadically in most other domestic species (Goldschmidt &Hendrick, 2002). Cutaneous hemangiomas are common in the dog, when compared to primary canine cutaneous hemangiosarcomas (Hargis et al., 1992). Hemangiosarcoma is one of the most challenging and mysterious diseases encountered in veterinary practice. It is a malignant, aggressive tumour that arises from the mutated vascular endothelial cells (Murakami et al., 2001). Hemangiosarcoma represents up to 7% of all tumours and 12% to 21% of all mesenchymal neoplasms in dogs (Clifford et al., 2000; Smith, 2003; Schultheiss, 2004; Thamm, 2007).

Two forms of the hemangiosarcomas i.e., visceral and dermal forms exists. In the visceral form, the most frequently affected organs were spleen and liver (Day et al., 1995; Withrow & MacEwen, 2001; Hristov et al., 2007) and rarely seen in the retroperitoneal organs like adrenal glands, kidneys and ureters (Wang & Su, 2001; Liptak et al., 2004) and uterus (Murakami et al., 2001). Whereas in dermal form, most common predilection sites are ventral abdomen, prepuce and pelvic limbs (Ward et al., 1994). In the breeds like Beagle, blood hound, English Pointer and Dalmatian, dermal form is commonly seen. German shepherds, Golden and Labrador retrievers, Schnauzers and Maltese breeds are also overrepresented in many case series (Smith, 2003; Hidaka et al., 2006; Hristov et al., 2007).

2 Materials and Methods

The tumour masses were surgically excised under general anaesthesia. The masses were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sections were taken with thickness of 5 um and stained with hematoxylin and eosin for histopathological examination.

3 Results and discussion

In the present study, vascular endothelial cell tumours arising from the cutaneous blood vessels were noticed clinically in three dogs out of twenty dogs affected with cutaneous tumours during the period from august 2015 to December 2015. Out of three cases, two were noticed in male cocker spaniels and one in male Labrador with a history of decreased appetite and weight loss.

3.1 Case 1

A ten year old male cocker spaniel dog was presented with a firm reddish tumour mass located on the skin near the ventral region of abdomen (Figure.1). Cut section of the mass revealed severe oozing of the blood.

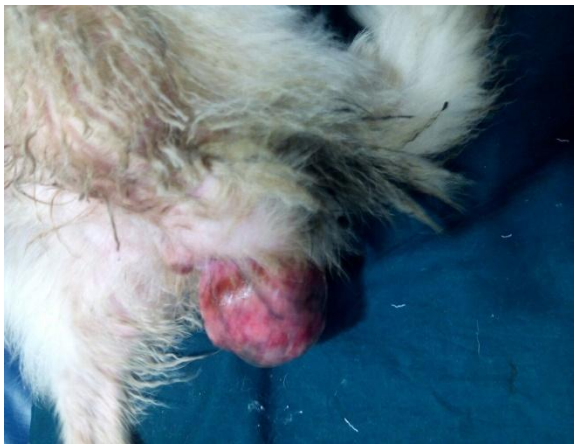

Figure 1 Note reddish mass under the abdomen in cocker spaniel dogs

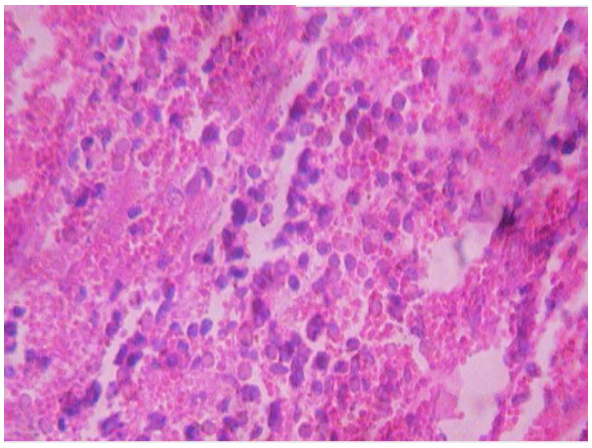

Figure 2 Note immature endothelial cells forming blood filled irregular vascular spaces

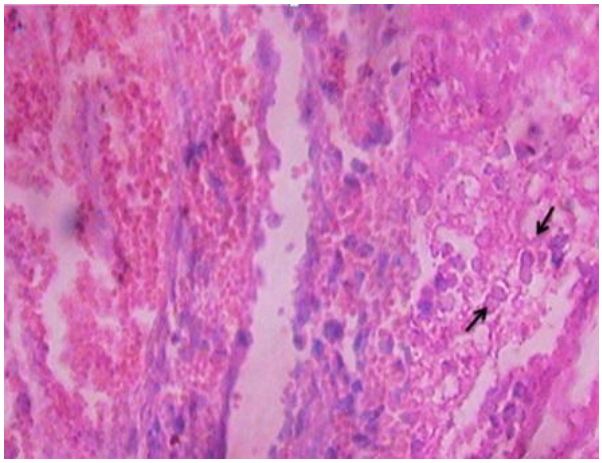

Figure 3 Section showing budding of blastomyces organisms (arrows) along with proliferated endothelial cells forming blood filled vascular spaces

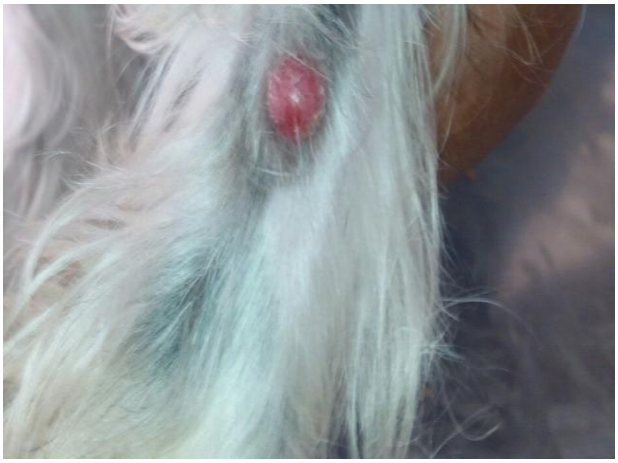

Figure 4 Note note red colored mass on the hindlimb in cocker spaniel

On microscopic examination, pleomorphic endothelial cells forming irregular vascular spaces containing variable amounts of blood were noticed (Figure.2). The immature endothelial cells were plump to spindle in shape with oval to round nuclei and it was diagnosed as hemangiosarcoma based on histopathological features. These features of hemangiosarcoma in the present study were similar to the features reported by previous authors (Park et al., 2008; Palanivelu et al., 2013). Further, in addition to proliferating endothelial cells, fungal infection with blastomyces species (Figure.3) was also observed as a secondary infection in our study.

3.2 Case 2

In a male cocker spaniel dog aged above six years, a soft dark red coloured mass was observed on the hindlimb (Figure.4). The mass revealed the presence of numerous greatly dilated blood filled vascular spaces lined by single layer of endothelial cells (Figure.5).The neoplastic endothelial cells have vesicular nuclei with eosinophilic cytoplasm. Based on microscopic lesions, it was diagnosed as cavernous hemangioma. These lesions are in accordance with the findings of Hargis et al. (1992) and Balachandran et al. (2014).

3.3 Case 3

A surgically excised soft greyish red coloured mass located on the ventral abdomen in six years old Labrador retriever was referred to the Department of Veterinary Pathology for histopathological findings. Histopathological examination revealed numerous vascular spaces lined by endothelial cells along with round to polymorphic fat cells having eccentrically placed nucleus (Figure.6 and Figure.7) and these findings were identified as lipohemangioma.

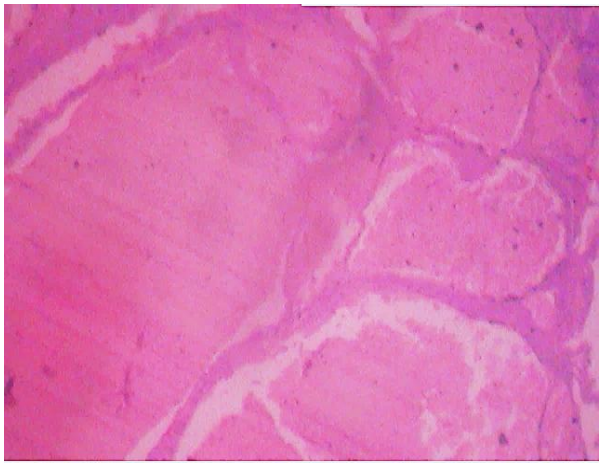

Figure 5 Section showing numerous greatly dilated blood filled vascular spaces lined by single layer of endothelial cells

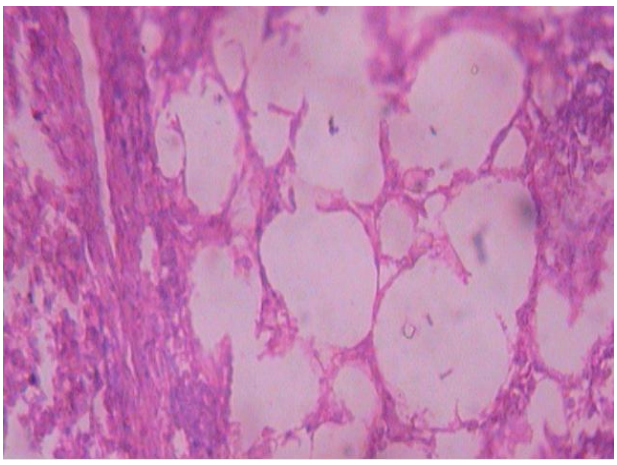

Figure 6 Note pleomorphic fat cells with eccentrically placed flattened nucleus

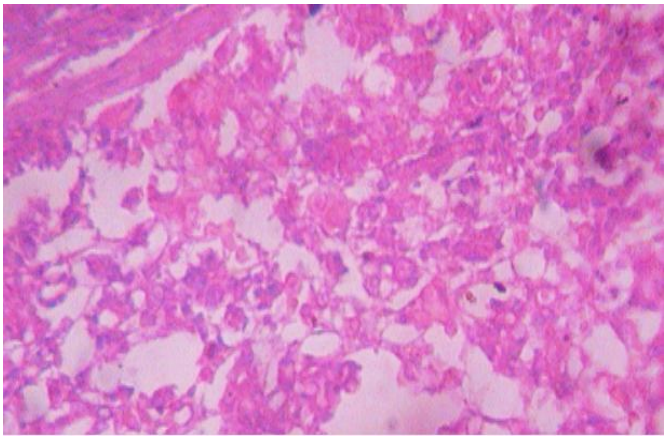

Figure 7 Note numerous vascular spaces containing RBCs along with immature lipocytes.

The features of mixed tumour of lipoma and hemangioma are in accordance with the earlier reports (Goldschmidt & Schofer, 1992; Palanivelu et al., 2013). Tumours occurring most commonly on the ventral abdomen and pelvic limbs were in agreement with a report of 13 Beagles, in which the skin of the lower abdomen and preputial area seemed to be most commonly affected (Culbertson, 1982). In the present study all the three cases were noticed in sparsely haired areas near the abdomen and on legs, for which the possible aetiology might be solar radiation as per the previous reports (Hargis et al., 1992; Ward et al., 1994).

Conclusion

In the present study, two benign and one malignant cutaneous vascular tumour were noticed and poor prognosis was observed in the case of malignant neoplasm i.e., hemangiosarcoma. Malignant tumours arising from the blood vessels are highly metastatic and aggressive and their prognosis is very poor. Early detection and treatment with surgery and chemotherapy prolongs the survival times. Histopathological examination was the most reliable method for diagnosis of cutaneous tumours.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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ASSESSMENT OF BRUCELLOSIS KNOWLEDGE, ATTITUDE AND PRACTICE AMONG VETERINARIANS IN INDIA

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KEYWORDS

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ABSTRACT

Brucellosis is considered as one of the major zoonotic infections worldwide. However, there is paucity of studies on knowledge, attitude and practice level of high risk groups like veterinarians in India. This study attempted to address this gap by conducting cross-sectional survey in four states of India (Assam, West Bengal, Uttar Pradesh and Punjab states). The data was collected from 160 veterinarians through the self-administered pre-tested questionnaire. There exist wide differences in knowledge level on brucellosis among veterinarians across the surveyed states. In all the studied states less than 12% of the veterinarians were fully aware on the nuances of Brucellosis Control Programme implemented in India indicating the need for appropriate intervention to achieve the intended targets. The knowledge, attitude and practice Index of majority of the veterinarians ranged between 74 to 85, 58 to 71 and 86 to 100, respectively. The knowledge on brucellosis is significantly correlated with the prevalence in the states (except West Bengal) indicating veterinarians in higher brucellosis prevalence states have better knowledge. There exists significant difference across the states in knowledge and attitude level on brucellosis indicating the need of imparting appropriate training to upgrade knowledge and to build positive attitude among veterinarians.

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1 Introduction

Brucellosis is regarded as one of the major zoonotic infections worldwide. More than 500 000 new cases occur annually but with an uneven global distribution (Skalsky et al., 2008). It remains an uncontrolled problem in regions of high endemicity such as Mediterranean, Middle East, Africa, Latin America and parts of Asia (Refai, 2002). Species infecting domestic animals are *Brucella melitensis* (goats and sheep), *B. suis* (pigs), *B. abortus* (cattle and bison), *B. ovis* (sheep), and *B. canis* (dogs) (Gull & Khan, 2007).

In India, bovine brucellosis is widespread and appears to be on the increase in recent times, perhaps due to increased trade and rapid movement of livestock (Renukaradhya et al., 2002). The increase in bovine brucellosis increases the risk of spread of the disease to veterinary professionals, especially, the field veterinarians who treats the animals on the daily basis. Brucellosis prevalence varied widely across the livestock and human population in different states of India. The sero-prevalence of brucellosis in milch goats and humans was 11.45% and 2.97%, respectively (Kapoor et al., 1985). In Gujarat state, 8.5% prevalence of *Brucella* agglutinins was recorded in human cases (Panjarathinam & Jhala, 1986). The various levels of brucellosis sero-prevalence across the species and states in India are reported by researchers (Rajkhowa et al., 2005; Londhe et al., 2011; Ramesh et al., 2013).

Brucellosis causes considerable economic loss in livestock and humans. In livestock, brucellosis causes reduced productivity, abortion, and weak offspring. The death of animal may also occur as a result of acute metritis, followed by retained fetal membranes (Radostitis et al., 2000). In humans, bone and joint involvement is the most frequent complication of brucellosis (Mousa et al., 1987). Besides affecting the healthy life in humans, brucellosis causes considerable financial burden of the family like increased cost of hospital treatment, cost of drugs, patient out-of pocket treatment expenses, loss of work or income loss due to illness, and Disability Adjusted Life Year (DALY) burden. During pregnancy brucellosis poses a substantial risk of spontaneous abortion or intrauterine transmission of infection to the infant (Hajia et al., 2009). The veterinary professionals who treat animals on daily basis form the high risk group for brucellosis. If the set practices are not followed by the veterinarians especially for the brucellosis suspected cases, might result in infection of this deadly disease to the veterinarians. In the long-run it aids the spread to their partners (Shome et al., 2014). Thus, brucellosis prevention, control and eradication are very important for any country to protect humans and animals. Considering the economic losses in livestock and zoonotic importance of the disease, Government of India implemented Brucellosis Control Programme (Brucellosis CP) during 2010 for mass screening followed by vaccination in areas where incidence of disease is high. The aim of Brucellosis-CP is to reduce economic loss and to reduce impact of the disease on human health besides mitigating the loss due to infection in animals. The success of public health programme like Brucellosis CP implemented in India depends on the Knowledge, Attitude and Practice (KAP) of the field level implementing officials like veterinarians. There is paucity of studies on KAP and hence this study attempted to address this gap by conducting cross-sectional survey among veterinarians in important states of Northern India.

2 Materials and Methods

2.1 Study area

The study was performed in four states of India (Assam, West Bengal, Uttar Pradesh and Punjab) during 2013-14. Assam is in north-eastern region with a livestock population of 19 million; West Bengal is in eastern part of India with livestock population of 30 million; Uttar Pradesh is located in northern India with livestock population of 68 million; and Punjab is in the north-western region of India with livestock population of 8 million (DAHD & F, 2015).

2.2 Sample size

A two stage proportionate sampling technique was adopted for the primary survey to assess the KAP levels of veterinary professionals. In the first stage, four states in India viz., Assam, West Bengal, Uttar Pradesh and Punjab were selected randomly. In the second stage, the proportionate sampling technique was adopted to select the number of respondents from each of the selected states based on the number of veterinarians working in the state. The selection of the sample size for this study was based on table developed by Bartlett et al., 2001. The sample size for a population 4,000 to 6,000 ranges from 198 to 209 for 0.03 margin error and 0.01 alpha. Accordingly, based on number of veterinarians working in the states (5123), the samples targeted for survey were 200. Out of 200 samples targeted, 160 participated and filled the knowledge component of the questionnaire, whereas, only 148 and 132 veterinarians participated in the attitude and practice component of the questionnaire, respectively. For operational feasibility, the survey was conducted during one day orientation programme on Brucellosis CP organized by the Animal Husbandry Department of states along with authors institute with the support of DAHD&F, India. Hence, for the present study the data was collected from the veterinarians who attended this orientation.

2.3 Questionnaire Development and Administration

The authors institute organizes various epidemiological trainings on brucellosis for the benefit of field veterinarians in India. During these trainings the KAP questionnaires developed by the authors were pre-tested and modified accordingly. Thus, a standard, pre-tested, structured questionnaire developed was self-administered to veterinarians working in different states of India during the main survey.

Table 1 Description and format of questions used to collect information on KAPs from the respondents.

Topic	Details	Question format
Knowledge statements **A. *Brucella* organism**		
About the Brucella organism	Participants have to ascertain the type of organism, mode of invasion, excretion and complexity of the organism.	Closed ended (Yes /No)
Clinical signs of brucellosis	Participants have to ascertain the disease symptoms in male and female animals like occurrence of abortion in any point of pregnancy, abortion occurs mostly during third trimester with leathery placenta and symptoms in male animals are epididymitis, orchitis and hygroma.	Closed ended (Yes /No)
Source of infection in livestock	Participants have to identify the possible source of infection like aborted fetuses, afterbirth and vaginal discharge. Questions were separately formulated to investigate other source of infection like pastures, fodder and water. Questions on dam to calf infection and using the semen straw produced from infected bulls were also provided.	Closed ended (Yes /No)
Causes of brucellosis in humans	Participants have to identify the sources of infection like physical contact, assisting parturition, contact with fetal membrane, drinking raw milk etc.	Closed ended (Yes /No)
Vaccination against brucellosis	Participants have to identify the type of vaccine against brucella, about age and sex of the animals to be vaccinated, any booster dose requirement, and immunity level of the vaccine in the animals etc.	Closed ended (Yes /No)
Brucellosis Control Programme (Brucellosis CP)	Participants have to identity the correct statements related to Brucellosis CP.	Closed ended (Yes /No)
Others aspects of brucellosis	Questions were also developed to assess the knowledge level on the susceptibility of breeds, age groups and hazard nature for the professionals.	Closed ended (Yes /No)
Attitude statements	Attitude towards the importance of brucellosis, its spread, control programme, disease tagging to monitor disease and farmers participation in the control programme	Likert scale (agree/ neutral/ disagree)
Practice statements	Questions were developed to assess the practice of usage of protective gears by veterinarians, sending suspected samples to labs, advisory service to farmers to control brucellosis at farm level	Closed ended (Yes /No)

Table 2 Knowledge on different aspects of brucellosis and Brucellosis CP in the study states.

	Assam		Punjab		Uttar Pradesh		West Bengal		Pooled	
Knowledge statements	No. of Respondents	%	No. of Respondents	%	No. of Respondents	%	No. of Respondents	%	No. of Respondents	%
About *Brucella* organism										
4/4	15	60	8	25	17	27	11	28	51	32
3/4	7	28	19	59	30	48	23	57	79	49
2/4	3	12	3	10	12	19	6	15	24	15
Clinical signs on brucellosis										
3/3	15	60	27	84	48	76	32	80	122	76
2/3	8	32	4	13	15	24	7	18	34	21
***Brucella* Infection in livestock**										
4/4	6	24	24	75	36	57	30	75	96	60

3/4	13	52	6	19	21	33	4	10	44	28
2/4	6	24	2	6	6	10	4	10	18	11
Causes/chances(zoonotic potential) of brucellosis to humans										
7/7	5	20	4	13	7	11	6	15	5	20
6/7	8	32	22	69	35	56	18	45	8	32
5/7	7	28	5	16	10	16	6	15	7	28
4/7	2	8	1	3	7	11	6	15	2	8
3/7	2	8	0	0	1	2	2	5	2	8
2/7	0	0	0	0	1	2	1	3	0	0
Vaccination against brucellosis										
6/6	2	8	21	66	17	27	27	68	67	42
5/6	17	68	9	28	23	37	11	28	60	38
4/6	5	20	1	3	12	19	0	0	18	11
3/6	1	4	1	3	8	13	0	0	10	6
2/6	0	0	0	0	2	3	1	3	3	2
Brucellosis Control Programme (Brucellosis-CP)										
4/4	3	12	3	9	5	8	5	13	16	10
3/4	4	16	3	9	9	14	13	33	29	18
2/4	8	32	13	41	15	24	12	30	48	30
Other aspects of brucellosis										
3/3	1	4	7	22	43	68	22	55	73	46
2/3	12	48	24	75	16	25	14	35	66	41

Table 3 Knowledge Index on brucellosis in different surveyed states.

Knowledge Index	**Assam**		**West Bengal**		**Uttar Pradesh**		**Punjab**		**Pooled**	
	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative
19 to 30	-	0	1(2.5)	1(2.5)	1(1.6)	1(1.6)	-	0	2(1.3)	2(1.3)
30 to 41	-	0	-	1(2.5)	-	1(1.6)	-	0	-	2(1.3)
41 to 52	1(4.0)	1(4.0)	1(2.5)	2(5.0)	-	1(1.6)	1(3.1)	1(3.1)	3(1.9)	5(3.1)
52 to 63	2(8.0)	3(12.0)	3(7.5)	5(12.5)	3(4.8)	4(6.3)	-	1(3.1)	8(5.0)	13(8.1)
63 to 74	6(24.0)	9(36.0)	2(5.0)	7(17.5)	16(25.4)	20(31.7)	3(9.4)	4(12.5)	27(16.9)	40(25.0)
74 to 85	11(44.0)	20(80.0)	14(35.0)	21(52.5)	30(47.6)	50(79.4)	22(68.8)	26(81.3)	77(48.1)	117(73.1)
85 to 96	5(20.0)	25(100.0)	19(47.5)	40(100.0)	13(20.6)	63(100.0)	6(18.8)	32(100.0)	43(26.9)	160(100.0)
Total	25(100.0)		40(100.0)		63(100.0)		46(100.0)		160(100.0)	

Figures in parenthesis indicates percentage to the total

Table 4 Results of attitude levels on brucellosis in different states.

Attitude statements	Assam			West Bengal			Uttar Pradesh			Punjab			Pooled		
	A	N	DA	A	N	DA	A	N	DA	A	N	DA	A	N	DA
1. It is a very important disease causing huge economic loss to any other disease in cattle	17 (70.8)	6 (25.0)	1 (4.2)	23 (59.0)	2 (5.1)	14 (35.9)	38 (71.7)	5 (9.4)	10 (18.9)	21 (67.7)	5 (16.1)	5 (16.1)	99 (67.3)	18 (12.2)	30 (20.4)
2.It is the top most bacterial disease in outbreak reporting in India	7 (29.2)	11 (45.8)	6 (25.0)	12 (32.4)	2 (5.4)	23 (62.2)	8 (15.7)	17 (33.3)	26 (51.0)	10 (33.3)	10 (33.3)	10 (33.3)	37 (26.1)	40 (28.2)	65 (45.8)
3. The chance of brucellosis spread in India is fast compared to western countries	19 (82.6)	4 (17.4)	0 (0)	30 (81.1)	2 (5.4)	5 (13.5)	41 (78.8)	8 (15.4)	3 (5.8)	29 (96.7)	1 (3.3)	0 (0)	119 (83.8)	15 (10.6)	8 (5.6)
4. The disease may spread from bovine to sheep and goat	9 (37.5)	7 (29.2)	8 (33.3)	22 (61.1)	7 (19.4)	7 (19.4)	32 (61.5)	10 (19.2)	10 (19.2)	23 (82.1)	0 (0)	5 (17.9)	86 (61.4)	24 (17.1)	30 (21.4)
5. The disease may spread from sheep and goat to bovine	8 (36.4)	7 (31.8)	7 (31.8)	21 (58.3)	8 (22.2)	7 (19.4)	33 (62.3)	7 (13.2)	13 (24.5)	20 (69.0)	2 (6.9)	7 (24.1)	82 (58.6)	24 (17.1)	34 (24.3)
6. The GOI implemented control programme on brucellosis is fool proof and will be successful	14 (63.6)	8 (36.4)	0 (0)	26 (74.3)	5 (14.3)	4 (11.4)	26 (54.2)	15 (31.3)	7 (14.6)	6 (20.0)	23 (76.7)	1 (3.3)	72 (53.3)	51 (37.8)	12 (8.9)
7. The test and slaughter policy will yield better results than vaccination	10 (40.0)	6 (24.0)	9 (36.0)	17 (45.9)	9 (24.3)	11 (29.7)	18 (35.3)	13 (25.5)	20 (39.2)	19 (63.3)	7 (23.3)	4 (13.3)	64 (44.8)	35 (24.5)	44 (30.8)
8. Tagging will be helpful for tracking the disease spread in Indian condition	17 (68.0)	5 (20.0)	3 (12.0)	24 (68.6)	6 (17.1)	5 (14.3)	40 (78.4)	6 (11.8)	5 (9.8)	23 (76.7)	5 (16.7)	2 (6.7)	104 (73.8)	22 (15.6)	15 (10.6)
9. Marginal, small and landless farmers have the capacity to pay for vaccination if it is priced?	3 (12.0)	5 (20.0)	17 (68.0)	7 (18.9)	3 (8.1)	27 (73.0)	9 (17.6)	8 (15.7)	34 (66.7)	11 (36.7)	4 (13.3)	15 (50.0)	30 (21.0)	20 (14.0)	93 (65.0)
10. Livestock insured farmers will be forthcoming than non-insured farmers for vaccination programme	11 (45.8)	4 (16.7)	9 (37.5)	25 (67.6)	4 (10.8)	8 (21.6)	24 (48.0)	17 (34.0)	9 (18.0)	11 (36.7)	13 (43.3)	6 (20)	71 (50.4)	38 (27.0)	32 (22.7)

Some of the respondents did not answered some of the attitude statements hence there is difference in number of respondents across the statements; A-agree, N-Neutral, DA-disagree

Table 5 Attitude Index on brucellosis in different surveyed states.

Attitude Index	Assam		West Bengal		Uttar Pradesh		Punjab		Pooled	
	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative
6 to 19	-	0	1(2.6)	1(2.6)	-	0	1(3.3)	1(3.2)	2(1.4)	2(1.4)
19 to 32	-	0	-	1(2.6)	1(1.9)	1(1.9)	-	1(3.2)	1(0.6)	3(2.0)
32 to 45	-	0	1(2.6)	2(5.1)	1(1.9)	2(3.8)	1(3.3)	2(3.3)	3(2.0)	6(4.0)
45 to 58	11(44.0)	11(44.0)	4(10.3)	6(15.4)	4(7.5)	6(11.3)	2(6.7)	4(12.9)	21(14.2)	27(18.2)
58 to 71	12(48.0)	23(92.0)	19(48.7)	25(64.1)	32(60.4)	38(71.7)	15(50.0)	19(61.3)	78(52.6)	105(70.8)
71 to 84	2(8.0)	25(100.0)	10(25.6)	35(89.7)	13(24.5)	51(96.2)	12(40.0)	31(100.0)	37(25.0)	142(95.9)
84 to 97	-	0	4(10.3)	39(100.0)	2(3.8)	53(100.0)	-	31(100.0)	6(4.1)	148(100.0)
Total	25(100.0)		39(100.0)		53(100.0)		31(100.0)		148(100.0)	

Figures in parenthesis indicates percentage to the total

Table 6 Results of practice levels by veterinarians to prevent the spread of brucellosis in the study states.

Practice statements	Assam		West Bengal		Uttar Pradesh		Punjab		Pooled	
	Practiced	Not practiced	Practiced	Not practiced	Practiced	Not practiced	Practiced	Not practiced	Practiced	Not practiced
1. Obtain complete clinical history about the *Brucella* suspected animal	17(70.8)	7(29.2)	29(80.6)	7(19.4)	37(77.1)	11(22.9)	28(93.3)	2(6.7)	111(80.4)	27(19.6)
2. Gloves, goggles and mask should be used before attending parturition	21(84.0)	4(16.0)	18(52.9)	16(47.1)	33(68.8)	15(31.3)	22(75.9)	7(24.1)	94(69.1)	42(30.9)
3. Send clinical samples of suspected animals for confirmatory diagnosis	21(87.5)	3(12.5)	27(75.0)	9(25.0)	19(40.4)	28(59.6)	27(90.0)	3(10.0)	94(68.6)	43(31.4)
4. Advice farmers about the proper disposal of infected materials	23(95.8)	1(4.2)	31(86.1)	5(13.9)	42(89.4)	5(10.6)	28(96.6)	1(3.4)	124(91.2)	12(8.8)
5. Advice farmers to buy animals after assessing the history of brucellosis symptoms	19(82.6)	4(17.4)	21(61.8)	13(38.2)	38(80.9)	9(19.1)	20(69.0)	9(31.0)	98(73.7)	35(26.3)
6. Advice farmers not to use *Brucella* infected animal for natural service	21(87.5)	3(12.5)	31(86.1)	5(13.9)	43(91.5)	4(8.5)	28(96.6)	1(3.4)	123(90.4)	13(9.6)

Table 7 Practice Index on brucellosis in different surveyed states.

Practice Index	Assam		West Bengal		Uttar Pradesh		Punjab		Pooled	
	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative	No. of respondents	Cumulative
16 to 30	-	0	-	0	1(2.2)	1(2.2)	-	0	1(0.8)	1(0.8)
30 to 44	2(8.0)	2(8.0)	-	0	3(6.5)	4(8.7)	-	0	5(3.8)	6(4.5)
44 to 58	2(8.0)	4(16.0)	1(3.2)	1(3.2)	4(8.7)	8(17.4)	2(6.7)	2(6.7)	9(6.8)	15(11.4)
58 to 72	3(12.0)	7(28.0)	11(35.5)	12(38.7)	10(21.7)	18(39.1)	4(13.3)	6(20.0)	28(21.2)	43(32.6)
72 to 86	8(32.0)	15(60.0)	6(19.4)	18(58.1)	13(28.3)	31(67.4)	10(33.3)	16(53.3)	37(28.0)	80(60.6)
86 to 100	10(40.0)	25(100.0)	13(41.9)	31(100.0)	15(32.6)	46(100.0)	14(46.7)	30(100.0)	52(39.4)	132(100.0)
Total	25(100.0)		31(100.0)		46(100)		30(100.0)		132(100.0)	

Figures in parenthesis indicates percentage to the total

The questionnaire comprise basic demographic information viz., name, designation, working region, age, education qualification, years of service etc. besides various statements on brucellosis to assess the KAP level of veterinarians. The description and formation of questions to collect information on KAP parameters is presented in Table 1. The knowledge component of the questionnaire were divided into *Brucella* organism, clinical signs, source of infection in livestock, causes of brucellosis in humans, vaccination, Brucellosis CP and other aspects. The number of questions varied in different components/groups of the knowledge statement. The questions on KAP parameters comprised of both negative and positive statements. The negative statements were included as a part of the knowledge/attitude evaluation in order to differentiate the assessment clearly.

Based on the statement type (positive and negative) the scores were awarded. The positive statements were awarded larger score compared to negative statement. The two point assessment for knowledge (yes; no) and practice statements (practiced; not practiced), and three point Likert scale (agree; neutral and disagree) for attitude was considered. The scores for positive statements are yes (1) and no (0) and for negative statements yes (0) and no (1) to assess knowledge level. Similarly, for evaluating the practice/adoption level each statement was awarded one or zero based on practice/not practiced by the individual veterinarians. For attitude assessment, the scores for positive statements were (Agree-3; Neutral-2 and Disagree-1) and for negative statements the scores were (Agree-1; Neutral-2; Disagree-3). The information on different aspects of brucellosis was collected from the veterinarians with their oral consent to participate in the survey.

2.4 Statistical analysis

Indexes for knowledge, attitude and practice were calculated for the individual respondents and overall for the surveyed states instead of raw scores for easy comprehension. The Index was calculated based on Rahman, 2007.

a) Individual Knowledge Index (KI) was calculated by

KI = (AK/MK)*100
Where, KI = Knowledge Index, AK = Actual scoring of respondents for knowledge statements, MK = Maximum score for knowledge statements

b) Overall Knowledge Index was calculated by
OKI = ∑KI/n
Where, ∑ KI = Summation of individual Knowledge Index, n = Total number of respondents, Similar formulae were used to calculate individual and overall Practice and Attitude index.

c) Sturges rule was employed for classifying the number of classes in knowledge, attitude and practice Index.

d) Correlation (r) was used to determine the relationship between Index (KI or AI or PI) with various demographic parameters like age, number of years of service, education and also with prevalence level of brucellosis in their respective states.

e) The non-parametric Kruskal -Wallis Test was employed to compare the levels of knowledge, attitude and practice Index across the states surveyed

$$KW = \frac{12}{n(n+1)} \sum_{j=1}^{k} \frac{R^2}{n_j} - 3(n+1)$$

Where,

KW = Kruskal-Wallis value
k = number of groups
NJ = size of the *j*th group
RJ = rank sum for the *j*th group
n = total sample size

3 Results

3.1 Socio-demographic characteristics of the respondents

The age, education and experience details of the respondents in different surveyed states revealed that the overall median age and experience of the veterinarians was 44 years and 19 years, respectively.

3.2 Knowledge

Knowledge provides the basis for human behaviour and action. In the present study the knowledge of the veterinarians on brucellosis was assessed through set of statements on *Brucella* organism, clinical signs, and source of infection in livestock, causes of brucellosis in humans, vaccination, Brucellosis CP etc. The knowledge on various aspects across the states is summarised in Table 2. The state level results revealed that in Assam, 60% of the veterinarians had comprehensive knowledge (gave correct answers for all statements whereas it was less in other states (West Bengal, Uttar Pradesh, and Punjab) indicating differences in knowledge level on brucellosis in the surveyed states. The pooled results revealed that 32% (4/4) of the respondents gave correct answers for all statements on *Brucella* organism *per se* whereas, 49% (3/4), 15% (2/4), and 4% (1/4) of the respondents, gave 75%, 50% and 25% correct answers, respectively. The knowledge on clinical signs of brucellosis was generally high across the states, whereas, the knowledge level on source of infection in livestock varied across the state (Punjab and West Bengal (75%), Uttar Pradesh (57%) and Assam (24%)) implying the need for intervention to improve knowledge level of veterinarians.

In all the study states less comprehensive knowledge (gave correct answers to all the questions) on causes/chances of brucellosis was observed. On vaccine and vaccination statements, around 68% and 66% of the veterinarians from West Bengal and Punjab, respectively were completely aware, whereas, it was only less than 30% in other states indicating variation in knowledge level on vaccine and vaccination details against brucellosis. In the study area less veterinarians had comprehensive knowledge on Brucellosis CP indicating the wide knowledge gap and necessity of training the veterinarians on different aspects on Brucellosis CP. The results on the knowledge level on susceptibility of breeds, age groups and hazard nature for the professionals revealed that 68% and 55% of the veterinarians from Uttar Pradesh and West Bengal respectively were fully aware on these aspects.

3.3 Knowledge Index

The Knowledge Index (KI) in different states is summarised in Table 3. In all the surveyed states except West Bengal maximum number of the respondents was in 74-85 KI. In West Bengal, the maximum number of respondents was in 85-96 range indicating the better knowledge level in this state compared to other surveyed states.

3.4 Attitude

Positive attitude combined with comprehensive knowledge especially among the field veterinarians will help to bolster the brucellosis control initiatives of the Government. The attitude results revealed that across the study states more than two-third (67%) of the veterinarians agreed that brucellosis is a very important disease causing huge economic loss to any other disease in cattle (Table 4). There were wide differences in agreement across the states to the statement "brucellosis is the top most bacterial disease reported in the country" viz., Assam (30%), West Bengal (32%), Punjab (33%) and Uttar Pradesh (16%). In the study states, majority (84%) of respondents believed that the chance of brucellosis spread in India's rural setting is fast compared to western countries. For the disease transmission statement from sheep and goat to bovines and vice versa, irrespective of the state, majority of the respondents believed that both way transmissions take place in livestock. Higher proportion of veterinarians (Assam (64%), West Bengal (74%), and Uttar Pradesh (54%) except in Punjab state, opined that the Brucellosis CP implemented by Government of India will be successful in controlling the disease.

Though the test and slaughter policy for brucellosis disease control might yield better results than vaccination, majority of the respondents were divided on the issue. The majority (74%) of the veterinarians in the study states exhibited positive attitude towards tagging the diseased animal and tracking the same to control the brucellosis spread. The opinions were varied across the study states among veterinarians for the statement 'marginal, small and landless farmers have the capacity to pay for vaccination if priced? and "Livestock insured farmers will be forthcoming than non-insured farmers for vaccination programme" due to attitude differences (Table 4).

3.5 Attitude Index

The attitude index of the veterinarians revealed that majority (60%) of the veterinarians in Uttar Pradesh had between 58 to 71, whereas, it was 50%, 49% and 48% respondents in Punjab, West Bengal and Assam, respectively. The pooled results revealed that majority of the respondent's attitude ranged from 58-71 (Table 5).

3.6 Practice

Besides the knowledge and attitude levels, the practice of the veterinarians, especially, when handling the suspected brucellosis case is an important in controlling the disease. Majority of the veterinarians in the surveyed states obtain complete clinical history of the animals suspected with brucellosis, but still 7 to 29% of the respondents are not collecting complete history (Table 6). The proportion of veterinarians using gloves, goggles and mask during parturition varied across the states viz., Assam (84%), West Bengal (53%), Uttar Pradesh (69%) and Punjab (76%). Sending clinical samples of the suspected animals for confirmatory diagnosis is not practiced by all the veterinarians. Advising farmers on the proper disposal of infected materials is practiced by majority of the respondents across the states (Assam (96%), West Bengal (86%), Uttar Pradesh (89%) and Punjab (97%)). Similar results were obtained for the practice statements 'Advice farmers to buy animals after assessing the history of brucellosis symptoms' and 'Advice farmers not to use brucellosis infected animals for natural service'.

3.7 Practice Index

The practice index is very important since any deviation from the set procedure in treating the animals will widen the scope of infection and spread of brucellosis. In all the surveyed states the practice index levels among the veterinarians was less (Table 7). The cumulative practice levels of respondents revealed that in Uttar Pradesh two-third of the veterinarians had less than 86 index level, whereas in Assam, West Bengal and Punjab the proportion of respondents were 60%, 58% and 53%, respectively.

Table 8 Correlation between Knowledge, Attitude and Practice Index vis-à-vis other variables.

Attributes/ state	Assam			West Bengal			Uttar Pradesh			Punjab			Pooled		
	K	A	P	K	A	P	K	A	P	K	A	P	K	A	P
Age	0.11^{ns}	0.06^{ns}	-0.07^{ns}	-0.06^{ns}	-0.32^{ns}	0.03^{ns}	-0.18^{ns}	-0.36^{ns}	-0.21^{ns}	0.16**	0.02^{ns}	0.02^{ns}	-0.008^{ns}	-0.19^{ns}	-0.06^{ns}
Education	-0.28^{ns}	0.14^{ns}	0.20^{ns}	0.02^{ns}	-0.15^{ns}	0.07^{ns}	0.31^{ns}	0.20^{ns}	0.53^{ns}	0.15**	0.03^{ns}	0.2^{ns}	-0.01**	0.08^{ns}	0.25^{ns}
No. of years of service	0.11^{ns}	0.08^{ns}	0.05^{ns}	-0.05^{ns}	-0.24^{ns}	-0.02^{ns}	-0.51^{ns}	-0.37^{ns}	-0.28^{ns}	0.13**	-0.05^{ns}	0.05^{ns}	0.19^{ns}	-0.16^{ns}	-0.05^{ns}
Brucellosis Prevalence level in the state	0.00**	0.00**	0.00^{ns}	0.00^{ns}	0.00^{ns}	0.00^{ns}	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	-0.04**	-0.13**	0.043**

** indicates significance at 1% level of significance; ns=Non significant; K=Knowledge, A=Attitude, P=Practice

3.8 Correlation of knowledge level vs demographic variables and prevalence

No significant correlation was observed between the personal attributes and knowledge level of the veterinarians in all the states except Punjab (Table 8). In Assam, Uttar Pradesh and Punjab significant positive correlation ($p<0.01$) exists between brucellosis prevalence level in the state and the knowledge level of the veterinarians. The attitude and practice level of the respondents is not significantly correlated with the personal attributes in all the studied states of India ($p>0.01$), whereas, it is significantly correlated with prevalence level of Assam, Uttar Pradesh and Punjab ($p<0.01$). The results of non-parametric Kruskal–Wallis test revealed that there exists significant difference in knowledge and attitude level across the states ($p<0.01$), whereas, no significant difference in practice index across the states ($p>0.01$).

4 Discussions

Literature revealed that there is no information available on the knowledge, attitude and practice level of veterinarians on brucellosis in India for an important zoonotic disease like brucellosis, which has inherent difficulties in treatment and prevention due to various factors. In a federal country like India, the state administration play an important role in implementing various livestock and human resource development programs and hence, the state level analysis on KAP were carried out however, generalizing the results of this study should be approached cautiously due to region specific, small sample size and self reporting by the veterinarians. There were wide differences in knowledge about the type of organism, its nature of residence within host, its complexity in infecting various hosts. The differences might be due to various levels of knowledge updating, especially after joining field veterinary service, though it is needed among the risk groups to avoid zoonotic infections.

The knowledge on clinical signs is very important to suspect a case for brucellosis at field level. Around 60%, 80%, 84% and 76% of respondents from Assam, West Bengal, Uttar Pradesh and Punjab were aware of all the major clinical signs, but comprehensive knowledge should be imparted to field veterinarians. The results are in line with Adesiji et al. (2005), who reported that among 540 respondents who include butchers, herdsmen, meat vendors, milkers and veterinarians, only 19.6% are aware of the etiology, mode of transmission and prevention of brucellosis.

He also highlighted that there was a high level of awareness among the veterinarians than other groups studied. Same was observed in findings of Bilal et al. (1991) that out of 337 subjects examined for knowledge about methods and means of transmission of brucellosis, 309 (92%) were ignorant. There were wide differences in the knowledge on sources of *Brucella* infection in cattle and humans. Since *Brucella* is a zoonotic disease, the complete knowledge on infection in cattle and humans is essential to prevent its infection and spread to

livestock, veterinarians and their peer groups. It also helps the veterinarians to disseminate appropriate information to the farmers/farm animal handler to protect this deadly disease spread. The knowledge levels on vaccine and vaccination is very low in many states and it may be due to the fact that vaccine and vaccination policies in different countries are varying (S19 vs RB51 strain; Calf hood vs adult vaccination).

Brucellosis CP has been implemented in India since 2010, however, the knowledge level of the field veterinarians on Brucellosis CP was very low. Hence, detailed plan of Brucellosis CP as well as uniform guidelines on vaccination and surveillance has to be made available to the field veterinarians through the respective state governments for its effective implementation. Though the maximum numbers of respondents across the states were in the range of 74-85 knowledge index, there were few respondents with very low Knowledge Index. It implies the necessity of appropriate training on the theoretical and practical aspects on brucellosis disease to upgrade the knowledge levels of field veterinarians (Mazloomy et al., 2008). Hence, training might be the best option to achieve comprehensive knowledge on brucellosis among the veterinarians.

Across the states majority of the veterinarians agreed that brucellosis is a very important disease causing huge economic loss to any other disease in cattle. Though brucellosis may not inflict more direct losses like infectious diseases, but, its zoonotic nature will have wider social and economic ramifications and hence, majority respondents might believed that brucellosis causes huge economic loss. It is also evident from McDermott et al., (2013), that brucellosis is consistently ranked among the most economically important zoonoses globally. It is a 'multiple burdens' disease with economic impacts attributable to human, livestock and wildlife disease. Though Haemorrhagic Septicaemia (HS) disease in livestock is the top most bacterial disease reported in India (Benkirane et al., 2002), many veterinarians perceived that brucellosis as the top most bacterial disease reported implying lack of awareness about the macro disease scenario in the country. Majority of the respondents attitude towards the spread of brucellosis in India's rural setting is fast compared to developed countries and it might be due to lack of facilities for 'screening of all the animals for brucellosis' in India's rural setting and also due to policy ban on slaughter of cattle in majority of the states.

Also due to trade, rapid movement of livestock and preponderance of natural bull service in rural India (Lindahl et al., 2015) are the other compelling reasons to believe faster spread of brucellosis in India's rural setting. The majority of the respondents have positive attitude towards the tagging the diseased animal since it assists in tracking the animal. Majority of the respondents disagreed to the statement of pricing of the vaccine for marginal, small and landless farmers indicating the strong need for free public intervention to control brucellosis. Across the states the respondent's overall Attitude Index (58-71) was less than Knowledge Index (74-85) indicating more scope for changing the attitude through appropriate intervention.

Before handling the *Brucella* suspected animal, obtaining the complete history is important, but, in the surveyed states few veterinarians (7 to 29%) are not following this practice. Similarly, during the parturition majority of the veterinarians are not using all the major gadgets like gloves, goggles and mask. Majority of the respondents opined that they use only gloves mainly due to non-availability of other protective gears from the department. The results are on par with Hannah et al. (2011), who reported that only 21% of the total respondents used protective gears while dealing with cows having an abortion or with aborted materials. This shows there exists high risk of brucellosis spread among field veterinarians. Adesiji et al. (2005) also reported that presence of open wounds while handling diseased animal caused brucellosis in three veterinarians among 10 veterinarians surveyed.

Hence, there is a necessity to spread awareness among the veterinarians on the repercussions of non-using of protective gears besides ensuring the regular supply of these gears by the Government. Sending clinical samples of the suspected animals for confirmatory diagnosis is not practiced by all the veterinarians due to practical difficulties in field conditions. It may also accentuate the disease spread to many animals and humans in the long-run.

On the contrary, even if diagnosed, lack of national level compensation policy and ban on cow slaughter in various states of India may lead to distress sale and un-intended spread. Advising farmers on different facets of the brucellosis disease is not followed by all the veterinarians and hence, all the veterinarians should be stressed to educate farmers on the importance of proper disposal of infected materials; purchasing of the animals after assessing the history of brucellosis and advocating farmers to refrain from natural service from the brucellosis infected bulls in order to control the brucellosis spread. In all the surveyed states the practice index levels was low implying majority of the respondents do not follow the standard practices. Hence, there is scope for improvement on the part of Government as well as veterinary professionals for controlling brucellosis infection and spread in livestock and humans in India.

The KAP level is not significantly correlated with the personal attributes of the respondents in majority of the study states. In Punjab, positive correlation was observed for knowledge level and demographic variables (age, education and experience levels) of the veterinarians and brucellosis prevalence level. There exists significant difference within knowledge level and attitude level across the states and no significant difference within practice index across the states. The significant difference across states might be due to differences in institutional and extension activities taken up by the states to upgrade the KAP levels of veterinarians.

Conclusions

The comprehensive knowledge, attitude and set of practices in handling *Brucella* infected cases are not observed among the veterinarians. Hence, it warrants urgent intervention to sensitize the veterinarians through appropriate training and experiential learning techniques to upgrade the knowledge level and to build positive attitude. The field veterinarians need to be instructed to follow the set practices during the treatment of animals in order to avoid infection of brucellosis to themselves, peers and the society at large. The government investment is very much essential especially in providing the basic tools like gloves, goggles and mask to protect veterinarian and their staff from *Brucella* infection. The uniform guidelines for vaccination, surveillance, mechanism to handle diseased animals should also be disseminated to the field veterinarians appropriately to mitigate brucellosis infection and for the success of Brucellosis CP. Collective efforts in 'One Health' approach to improve knowledge, attitude and practice of veterinarians supported by appropriate government intervention will help to prevent the brucellosis infection and its spread among veterinarians, livestock farmers and related risk groups, thus making an initiative for one world one health approach.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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EFFECT OF MELATONIN ON MOBILITY AND VELOCITY PARAMETERS OF MITHUN (*Bos frontalis*) SEMEN PRESERVED IN LIQUID STATE ($5^{O}C$)

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Liquid semen preservation

ABSTRACT

Present study was conducted to evaluate the effect of melatonin (MEL) on velocity and motility of mithun semen by computer assisted sperm analyser. Semen ejaculates (n=25) were collected from matured mithun bulls (n=10) using trans-rectal massage method and were divided and grouped into six aliquots equally, extended with the standard Tris Egg Yolk Citrate semen diluent. Six groups for various treatments were prepare, these group were control - semen without MEL (Gr 1), 1mM (Gr 2), 2mM (Gr 3), 3Mm (Gr 4), 4mM (Gr 5:) and 5mM (Gr 6). Various parameter such as total motility, forward progressive motility, straight line velocity, curvilinear velocity, average path velocity, wobble, linearity, straightness, beat/cross frequency, amplitude of lateral head displacement and velocity of rapid, medium, slow and static were measured for 0-30h at 6 hrs interval at $5^{o}C$. The result observed that these mobility and velocity parameters were varied significantly ($p<0.05$) among the experimental periods and among the experimental groups. Further, MEL at 3 mM has significant ($p<0.05$) improvement in the mobility and velocity parameters than MEL at 1, 2, 4or 5 mM stored in *in- vitro* for up to 30 h of incubation. It was concluded that MEL 3 mM treated sperm has increased functional sperm structures faster to move and forward direction, probably improves the fertilization rate.

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1 Introduction

Mithun is a pride of North-Eastern hill region of India and is well adopted anatomically and physiologically at an altitude ranged from 300 –3000 meter MSL (Simoons, 1984). Latest livestock census (Livestock Census of India, 2012) revealed that population of mithun is reducing gradually due to lack of scientific strategy on reproduction and production areas. Required efforts should be undertaken from all quarters to preserve the germ plasm of mithun. Therefore, implementation of artificial breeding will enhance and improve the breeding strategy in mithun species.

For minimizing sperm metabolism and to maintain quality of the sperm, it was preserved at 5°C till the completion of study. But the sperm mobility and velocity parameters are reduced in the storage period. Antioxidants are present in semen to protect the sperm against free radical (Bilodeau et al., 2001; Bucak et al., 2008; Akhter et al., 2011). However, these antioxidants level are decreased during the process of extension and preservation process in liquid as well as in frozen state (Kumar et al., 2011). Therefore, supplementation (Shoae & Zamiri, 2008; Perumal et al., 2013) or feeding of the natural/synthetic antioxidants (Jayaganthan et al., 2013) could able to reduce the deleterious effect of oxidative and cryo stress during the process of semen preservation (Perumal et al., 2011a; Perumal et al., 2011b).

MEL is rhythmically secreted from the pineal gland and has an essential function in the circadian clock as well as the seasonal reproduction in mammalian species (Reiter, 1991). MEL and its metabolites also act as indirect powerful antioxidants to scavenge the free radicals (Reiter et al., 1998). Moreover, MEL has also potency two times as like of vitamin E in reaction against free radicals and reactive oxygen species (Pieri et al., 1994). The effect of anti-oxidant, MEL on ram (Casao et al., 2009; Ashrafi et al., 2011), boar (Hyun-Yong et al., 2006; Martin-Hildago et al., 2011), bull (Ashrafi et al., 2013); mithun (Perumal et al., 2013; Perumal et al., 2015), human (Du Plessis et al., 2010) and buffalo sperm (Li et al., 2012) revealed that it improves sperm mobility and velocity parameters of sperm in liquid storage. Perusal of literatures revealed that no information on the effect of MEL on the mobility and velocity parameters measured by CASA during liquid storage for extended period in mithun species. Hence, the objective of this study was designed to assess these parameters in semen samples extended with MEL at different concentration in liquid preservation for different incubation periods.

2 Material and Methods

2.1 Experimental Animals

Healthy matured mithun bulls (n=10) were selected. The experimental animals were maintained under same housing, feeding, watering and management systems. The feeding methods and feeds were followed uniformly as per the farm schedule. Semen ejaculates was collected through trans-rectal massage method from the matured mithun bulls. All the experimental protocols strictly followed the regulation proposed by Institutional Animal Care and Use Committee.

2.2 Semen collection and processing

The semen were ejaculates (n=25) and collected from the matured mithun bulls (n=10) but it should not more be than twice per week. The semen samples were placed in a water bath (37^{o}C) and analysed the routine semen quality parameters immediately after collection. The partially extended samples were then carried to the andrology laboratory in an insulated thermo flask filled with warm water (37^{o}C) for further processing. The ejaculates which have concentration >500 million ⁄ ml, individual motility >70%, mass activity >3+ and total abnormality <10%were accepted for further investigation.

Individual ejaculates were divided and grouped into six aliquots equally and extended with the TEYC extender with MEL. The groups are Gr 1: control; semen without MEL, Gr 2: 1mM, Gr 3: 2mM, Gr 4: 3mM, Gr 5: 4mM and Gr 6: 5mM. Extended semen samples were placed in the cold cabinet for 30 hrs at 5^{o}C. The velocity and motility parameters were estimated by CASA analyzer as per standard procedure in samples during storage of semen at 5^{o}C for 30 h.

2.3 Computer assisted sperm analysis

The casa parameters were measured by Hamilton Thorne Sperm Analyser. The sperm concentration was estimated with a phase-contrast microscope (Nikon, Eclipse 80i; 400× magnification) after the semen collection. 25 µL of semen was extended into 50-100 µL of TEYC and 5 µL of this extended semen was pipetted and loaded into a pre-warmed (37°C) dual chamber disposable Leja slide and was left to some time to settle on the mini-therm heating stage before the analysis. Parameter, total motility, forward progressive motility, straight line velocity, curvilinear velocity, average path velocity, wobble, linearity, straightness, beat/cross frequency, amplitude of lateral head displacement and velocity of rapid, medium, slow and static were measured for 0-30 h at 6 hrs interval at 5^{o}C. Minimum of 200 spermatozoa were assessed from at least different two drops of individual sample from each semen ejaculates. The objects were incorrectly observed as spermatozoa were manually deleted and final assessment was done for each semen sample. The values were analyzed using the computer software program. Significant differences were expressed at the values, $p<0.05$.

3 Results

The TM and PFM were significantly ($p<0.05$) higher in MEL added group as compared to untreated control group (Table 1). Furthermore, MEL 3 mM included samples have significantly ($p<0.05$) higher velocity and motility parameters.

Table 1 Mean (±S.E.) total motility (TM) and forward progressive motility (FPM) percentage for mithun semen following storage at 5°C for different storage time.

CASA parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Total Motility	Control	70.45 ± 2.30^{abC}	65.90 ± 2.53^{bcC}	47.95 ± 2.56^{abB}	35.55 ± 2.08^{aA}	31.95 ± 2.37^{aA}
	MEL 1 mM	72.90 ± 2.61^{abE}	68.95 ± 1.80^{cdD}	50.80 ± 1.96^{bcC}	41.00 ± 1.72^{bB}	36.90 ± 1.88^{bA}
	MEL 2 mM	75.20 ± 2.16^{Bd}	70.85 ± 2.09^{dD}	53.20 ± 2.22^{cdC}	44.50 ± 2.48^{bB}	39.90 ± 2.25^{bA}
	MEL 3 mM	82.70 ± 2.11^{cE}	75.50 ± 2.00^{eD}	56.75 ± 2.19^{dC}	51.65 ± 2.18^{cB}	45.40 ± 2.33^{cA}
	MEL 4 mM	72.85 ± 2.74^{abD}	62.30 ± 1.90^{abC}	46.15 ± 1.89^{aB}	35.15 ± 1.85 ^{aA}	31.80 ± 1.84^{aA}
	MEL 5 mM	68.75 ± 1.79^{aE}	61.70 ± 1.80^{aD}	46.25 ± 1.90^{aC}	33.35 ± 2.25^{aB}	27.95 ± 1.62^{aA}
Forward Progressive Motility	Control	30.05 ± 3.03^{abB}	22.10 ± 2.52^{aA}	19.85 ± 2.33^{abA}	19.10 ± 2.16^{abA}	17.50 ± 2.15^{bcA}
	MEL 1 mM	29.70 ± 2.61^{abB}	27.15 ± 2.10^{abB}	21.65 ± 2.47^{abA}	19.70 ± 1.88^{bA}	17.50 ± 2.30^{bcA}
	MEL 2 mM	31.75 ± 1.96^{bB}	31.40 ± 2.54^{bB}	19.90 ± 1.92^{abA}	19.00 ± 2.30^{abA}	19.30 ± 2.13^{cA}
	MEL 3 mM	41.00 ± 2.24^{cB}	38.65 ± 3.63^{cB}	23.70 ± 2.36^{bA}	19.60 ± 2.25^{bA}	19.15 ± 3.11^{cA}
	MEL 4 mM	26.10 ± 2.29^{aD}	24.70 ± 2.27^{abCD}	20.35 ± 2.64^{abBC}	17.30 ± 2.21^{abB}	12.45 ± 1.98^{abA}
	MEL 5 mM	29.10 ± 2.10^{abD}	24.05 ± 2.32^{aC}	18.35 ± 1.81^{aB}	15.00 ± 1.86^{aB}	11.05 ± 1.45^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$)

Table 2 Mean (±S.E.) different degree of velocity (percentage) of mithun sperm following storage at 5°C for different storage times.

Degree of Velocity	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Rapid Velocity	Control	59.10 ± 2.99^{abC}	51.25 ± 3.85^{aC}	36.20 ± 2.74^{aB}	28.05 ± 2.70^{abAB}	25.50 ± 2.66^{abA}
	MEL 1 mM	56.70 ± 3.00^{aC}	55.05 ± 3.96^{abC}	40.85 ± 2.94^{abB}	34.20 ± 2.67^{bcAB}	30.70 ± 2.21^{bcA}
	MEL 2 mM	67.05 ± 2.19^{bC}	64.55 ± 3.47^{bcC}	42.55 ± 3.50^{abB}	35.15 ± 2.54^{cAB}	32.50 ± 3.12^{cA}
	MEL 3 mM	81.35 ± 2.80^{cD}	69.95 ± 2.74^{cC}	47.50 ± 3.14^{bB}	43.75 ± 3.25^{dB}	33.80 ± 2.98^{cA}
	MEL 4 mM	59.70 ± 3.38^{abC}	54.60 ± 2.71^{abC}	35.25 ± 2.90^{aB}	27.20 ± 2.42^{aA}	23.15 ± 2.29^{aA}
	MEL 5 mM	62.85 ± 3.14^{abD}	53.80 ± 2.52^{aC}	36.60 ± 2.84^{aB}	24.30 ± 2.68^{aA}	19.25 ± 2.13^{aA}
Medium Velocity	Control	11.35 ± 2.37^{abBC}	15.15 ± 3.04^{cC}	12.45 ± 2.33^{BC}	8.25 ± 2.37^{abAB}	5.90 ± 1.79^{aA}
	MEL 1 mM	18.30 ± 3.88^{bB}	11.90 ± 3.03^{bcAB}	9.95 ± 2.71^{A}	6.06 ± 2.20^{aA}	6.70 ± 1.73^{aA}
	MEL 2 mM	10.55 ± 2.75^{abB}	3.60 ± 2.04^{aA}	12.90 ± 2.45^{B}	9.65 ± 2.99^{abAB}	10.05 ± 2.68^{abB}
	MEL 3 mM	5.55 ± 2.19^{aA}	12.65 ± 2.90^{bcB}	9.30 ± 2.95^{AB}	9.80 ± 2.43^{abAB}	11.65 ± 2.36^{bB}
	MEL 4 mM	13.15 ± 2.59^{abB}	7.90 ± 2.69^{abA}	11.05 ± 2.71^{AB}	8.55 ± 2.21^{abA}	8.85 ± 2.14^{abA}
	MEL 5 mM	7.20 ± 2.84^{aA}	7.95 ± 2.40^{abA}	10.10 ± 2.49^{AB}	12.65 ± 2.61^{bB}	8.45 ± 2.34^{abAB}
Slow Velocity	Control	15.45 ± 2.71^{bAB}	18.40 ± 2.44^{bAB}	20.30 ± 3.11^{abB}	16.75 ± 3.29^{AB}	12.45 ± 2.43^{aA}
	MEL 1 mM	13.60 ± 2.65^{bAB}	15.75 ± 2.70^{b}	18.15 ± 3.31^{ab}	16.85 ± 4.26	19.10 ± 3.17^{ab}
	MEL 2 mM	13.39 ± 2.49^{bAB}	8.35 ± 1.83^{aA}	20.15 ± 2.54^{abC}	16.35 ± 2.87^{BC}	17.85 ± 3.01^{abBC}
	MEL 3 mM	6.80 ± 1.83^{aA}	8.40 ± 2.41^{aAB}	15.35 ± 2.72^{aBC}	20.95 ± 3.50^{C}	19.00 ± 3.18^{abC}
	MEL 4 mM	14.60 ± 2.48^{bA}	16.20 ± 2.72^{bAB}	15.15 ± 2.74^{aA}	20.00 ± 3.23^{AB}	21.60 ± 3.52^{abB}
	MEL 5 mM	11.55 ± 2.45^{abA}	14.55 ± 2.61^{bAB}	24.80 ± 3.36^{bC}	22.45 ± 4.10^{BC}	22.40 ± 3.68^{bBC}
Static Motility	Control	14.15 ± 2.25^{bcA}	15.15 ± 1.98^{abA}	31.10 ± 3.78 ^{abB}	46.85 ± 3.79^{bC}	55.95 ± 2.86^{cD}
	MEL 1 mM	12.75 ± 2.49^{abA}	15.85 ± 2.83^{bA}	31.95 ± 3.38^{abB}	46.40 ± 2.61^{bC}	43.10 ± 3.39^{abC}
	MEL 2 mM	12.60 ± 3.16^{abA}	20.75 ± 2.40^{bcB}	24.30 ± 2.73^{aB}	37.70 ± 3.25^{abC}	38.55 ± 3.20^{abC}
	MEL 3 mM	7.10 ± 2.31^{aA}	9.50 ± 2.37^{aA}	25.50 ± 3.64^{aB}	27.85 ± 2.78^{aAB}	38.85 ± 3.36^{aC}
	MEL 4 mM	11.80 ± 1.99^{abA}	21.40 ± 3.05^{bcB}	37.65 ± 3.14^{bC}	44.20 ± 3.26^{bC}	46.45 ± 3.78^{abcC}
	MEL 5 mM	18.55 ± 2.26^{cA}	23.90 ± 2.73^{cA}	28.60 ± 3.21^{abA}	39.65 ± 4.22^{bB}	49.05 ± 3.88^{bcB}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$), Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$)

Table 3 Mean (±S.E.) average path velocity (VAP), straight line velocity (VSL) and curve linear velocity (VCL) of mithun sperm following storage at 5°C for different storage times.

Velocity Parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Average Path Velocity (VAP)	Control	122.33 ± 5.04^{abC}	106.51 ± 4.79^{aBC}	90.04 ± 4.04^{aAB}	89.35 ± 3.98^{AB}	87.88 ± 4.44^{abA}
	MEL 1 mM	126.80 ± 4.67^{abC}	118.79 ± 5.19^{abBC}	102.35 ± 4.53^{abAB}	99.75 ± 4.62^{AB}	89.70 ± 4.24^{abA}
	MEL 2 mM	131.88 ± 4.79^{bcC}	118.82 ± 5.57^{abBC}	100.97 ± 4.41^{abAB}	100.27 ± 5.21^{AB}	95.04 ± 4.42^{abA}
	MEL 3 mM	149.20 ± 4.42^{cC}	130.40 ± 5.35^{bBC}	109.15 ± 5.03^{bAB}	106.76 ± 5.29^{A}	98.10 ± 4.20^{bA}
	MEL 4 mM	110.83 ± 4.78^{aB}	98.67 ± 3.99^{aAB}	92.40 ± 4.39^{abA}	90.38 ± 4.08^{A}	87.36 ± 4.21^{abA}
	MEL 5 mM	110.03 ± 4.68^{aB}	98.18 ± 4.05^{aAB}	89.59 ± 4.36^{aAB}	88.55 ± 6.51^{AB}	83.95 ± 4.09^{aA}
Straight Line Velocity (VSL)	Control	85.47 ± 3.96^{abC}	76.27 ± 4.00^{abBC}	68.66 ± 4.32^{AB}	64.05 ± 4.16^{abAB}	58.80 ± 3.31^{aA}
	MEL 1 mM	85.98 ± 4.38^{abC}	79.23 ± 4.48^{abBC}	72.83 ± 4.01^{ABC}	67.97 ± 3.84^{abAB}	63.31 ± 4.36^{abA}
	MEL 2 mM	93.97 ± 4.95^{bcB}	80.57 ± 4.79^{abAB}	72.81 ± 4.77^{A}	71.95 ± 4.24^{bA}	64.52 ± 3.93^{abA}
	MEL 3 mM	103.18 ± 4.06^{cB}	92.78 ± 4.18^{bB}	75.39 ± 4.69^{A}	72.33 ± 4.01^{bA}	69.04 ± 3.89^{bA}
	MEL 4 mM	77.88 ± 4.14^{abB}	74.70 ± 4.24^{aAB}	66.22 ± 3.98^{AB}	65.20 ± 3.27^{abAB}	62.00 ± 3.25^{abA}
	MEL 5 mM	75.01 ± 4.25^{aB}	66.93 ± 4.14^{aAB}	64.90 ± 6.24^{AB}	59.31 ± 3.46^{aA}	57.35 ± 3.54^{aA}
Curve Linear Velocity (VCL)	Control	216.38 ± 7.84^{abB}	197.58 ± 7.48^{abAB}	191.38 ± 6.16^{abAB}	172.54 ± 5.03^{A}	169.73 ± 5.32^{A}
	MEL 1 mM	272.11 ± 5.26^{cB}	200.30 ± 6.37^{abA}	195.48 ± 5.92^{abA}	177.41 ± 8.19^{A}	175.06 ± 6.57^{A}
	MEL 2 mM	242.48 ± 6.36^{bcC}	224.64 ± 6.79^{bBC}	197.83 ± 5.55^{abAB}	187.81 ± 5.58^{A}	178.22 ± 5.79^{A}
	MEL 3 mM	242.65 ± 6.19^{bcB}	230.94 ± 7.27^{bB}	212.63 ± 6.75^{bAB}	188.24 ± 6.47^{A}	188.21 ± 4.63^{A}
	MEL 4 mM	206.05 ± 6.66^{abB}	191.29 ± 5.83^{abAB}	182.44 ± 6.00^{abAB}	176.85 ± 6.00^{AB}	166.58 ± 5.07^{A}
	MEL 5 mM	196.64 ± 7.54^{aB}	183.48 ± 5.01^{aAB}	173.90 ± 6.76 ^{aAB}	173.37 ± 5.81^{AB}	162.09 ± 7.70^{A}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$).

Proportionally the motility parameters were significantly ($p<0.05$) higher till 30 hrs of experimental period in the MEL 3mM treated group. Out of the five groups of MEL treated, MEL 4mM and MEL 5mM has significantly reduced TM and PFM. The motility parameters were increased gradually and significantly ($p<0.05$) from control to MEL 3mM group and then reduced in the MEL 4 and MEL 5 groups. The proportion of reducing TM and PFM were higher in MEL 4mM and MEL 5mM treated group as compared to other MEL treated groups.

In the present experiment, rapid velocity revealed that MEL included group has significantly higher percentage than untreated control group (Table 2). MEL 3mM treated group has significantly ($p<0.05$) higher rapid velocity than other treatment groups. Rapid velocity was increasing from 1 mM to 3 mM at maximum and reducing from 4 mM to 5 mM. Moreover, similar to TM and PFM, rapid velocity was reducing proportionally upto the experimental period (30 hrs of incubation).

The rapid velocity was positively and significantly correlated with PFM in all the experimental groups. The result revealed that there was a significant ($p<0.05$) difference among the experimental groups with regards to the VAP, VSL and VCL at different periods of incubation except at 24 hrs for VAP, 12 hrs of incubation for VSL and 24 and 30 hrs of incubation for VCL (Table 3). The velocity parameters (VAP, VSL and VCL) were significantly ($p<0.05$) higher in MEL 3mM treated group than the other treatment groups. These velocity parameters were significantly increased from 1mM to 3 mM and then decreased in 4 mM treated followed by MEL 5 mM. These velocity parameters were reduced over a period of the time during the experimental period. But the proportion was significantly higher in MEL high concentrated experimental groups (MEL 4 and 5 mM).

The result of ALH revealed that there was a significant ($p<0.05$) difference among the experimental groups in 6, 12 and 24 hrs of incubation (Table 4). Incubation period from 0 to 30 hrs, MEL 3mM was showing higher value than other treatment groups irrespective of significant or non-significant among the experimental groups.BCF revealed that there was a significant ($p<0.05$) difference among the experimental groups in 0, 24 and 30 hrs of incubation and significantly ($p<0.05$) higher in MEL 3mM followed by MEL 2 mM and least was in MEL 5 mM. The BCF value was increased from 1 mM to 3 mM and then decreased to 5 mM (Table 4).

Percentage of straightness revealed that there was a significant difference among the experimental groups at 6 hrs of incubation (Table 4).The MEL 3 mM was significantly($p<0.05$) higher among the experimental groups in 6 hrs of incubation. STR was significantly ($p<0.05$) differed among the experimental periods for all experimental groups except MEL 1 mM. But reduction of STR from 0 to 30 hrs of incubation was observed.

Table 4 Mean (±S.E.) amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) of mithun sperm following storage at 5°C for different storage times.

CASA Parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Amplitude of lateral head displacement (ALH)	Control	12.28 ± 3.85^{B}	8.60 ± 1.25^{aAB}	8.09 ± 1.24^{aAB}	8.07 ± 1.29^{aAB}	7.49 ± 1.10^{A}
	MEL 1 mM	9.01 ± 1.23^{B}	8.96 ± 1.28^{abB}	8.18 ± 1.26^{aAB}	8.09 ± 1.53^{aAB}	7.58 ± 1.33
	MEL 2 mM	10.58 ± 0.87^{B}	9.15 ± 1.29^{abA}	8.87 ± 1.07^{abA}	8.33 ± 1.20^{aA}	8.22 ± 1.27
	MEL 3 mM	10.90 ± 2.31^{B}	9.92 ± 1.20^{abB}	9.17 ± 1.47^{abAB}	9.11 ± 1.25^{abAB}	7.29 ± 1.23
	MEL 4 mM	11.57 ± 3.67	9.93 ± 1.43^{ab}	9.29 ± 1.14^{ab}	8.99 ± 1.22^{ab}	7.90 ± 1.33
	MEL 5 mM	13.21 ± 4.20	10.14 ± 1.24^{b}	10.01 ± 2.18^{b}	9.95 ± 2.08^{b}	7.72 ± 1.18
Beat cross frequency (BCF)	Control	28.32 ± 2.03^{aB}	26.96 ± 2.02^{B}	25.21 ± 2.38^{AB}	24.34 ± 2.32^{abcAB}	22.54 ± 2.30^{abA}
	MEL 1 mM	30.79 ± 2.71^{aC}	27.89 ± 2.35^{BC}	25.49 ± 2.30^{AB}	25.47 ± 1.96^{abcAB}	22.64 ± 1.79^{abA}
	MEL 2 mM	33.49 ± 6.71^{bC}	28.32 ± 2.19^{B}	27.59 ± 2.34^{A}	26.74 ± 1.99^{bcA}	24.32 ± 1.78^{bA}
	MEL 3 mM	43.31 ± 7.46^{cB}	29.54 ± 1.68^{A}	27.85 ± 2.41^{A}	27.30 ± 2.17^{cA}	25.79 ± 2.34^{bA}
	MEL 4 mM	28.26 ± 2.23^{aB}	26.56 ± 2.73^{BC}	24.98 ± 3.06^{AB}	23.18 ± 1.95^{abAB}	22.45 ± 1.95^{abA}
	MEL 5 mM	27.02 ± 2.29^{aC}	26.41 ± 2.20^{C}	23.60 ± 2.21^{BC}	21.91 ± 1.88^{aAB}	19.79 ± 1.54^{aA}
Straightness (STR)	Control	69.90 ± 2.81^{B}	69.40 ± 2.75^{abAB}	66.25 ± 2.46^{AB}	65.15 ± 2.29^{AB}	62.50 ± 3.36^{A}
	MEL 1 mM	69.95 ± 2.30	69.45 ± 2.81^{ab}	66.60 ± 2.26	65.30 ± 2.45	64.96 ± 3.89
	MEL 2 mM	72.00 ± 2.79^{B}	69.55 ± 2.82^{abAB}	68.45 ± 2.94^{AB}	64.80 ± 1.47^{A}	63.80 ± 2.31^{A}
	MEL 3 mM	72.50 ± 2.66^{B}	72.30 ± 2.70^{bB}	68.50 ± 2.59^{AB}	66.25 ± 1.88^{A}	64.20 ± 2.39^{A}
	MEL 4 mM	69.00 ± 3.13^{B}	66.50 ± 2.49^{abAB}	64.85 ± 2.58^{AB}	66.50 ± 2.36^{AB}	63.30 ± 1.88^{A}
	MEL 5 mM	68.95 ± 2.72^{B}	65.20 ± 2.43^{aA}	64.80 ± 2.13^{A}	64.35 ± 2.25^{A}	64.30 ± 2.03^{A}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$).

Similar to STR, LIN was significantly (p<0.05) differed among the experimental groups at 6 and 12 hrs of incubation at 5°C (Table 5). But the incubation period from 0 to 30 hrs, the MEL 3 mM group was significantly (6 and 12 hrs) higher among the experimental groups. The value of LIN was reduced significantly (p<0.05) from 0 to 30 hrs of incubation and it was lesser proportion in the MEL 3 mM or 2 mM treated groups and higher in MEL 4 mM or 5 mM treated groups. The WOB revealed that there was a significant difference was observed among the experimental groups in 0 and 6 hrs of incubation periods. Moreover, control and MEL 5 mM groups were significantly (p<0.05) differed in 0 hr of incubation. The value of WOB was reduced from 0 to 30 hrs of incubation for the group MEL 2 mM and MEL 4 mM whereas significant (p<0.05) difference was observed in other experimental groups (Table 5).

Table 5 Mean (±S.E.) linearity (LIN) and wobble (WOB)of mithun sperm following storage at 5°C for different storage times.

CASA parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Linearity (LIN)	Control	41.00 ± 3.08^{B}	37.75 ± 2.69^{aAB}	37.65 ± 2.60^{abAB}	36.35 ± 2.46^{AB}	35.40 ± 2.87^{A}
	MEL 1 mM	42.25 ± 3.91^{B}	39.60 ± 2.62^{aAB}	38.95 ± 2.70^{abAB}	37.50 ± 2.04^{AB}	35.35 ± 2.16^{A}
	MEL 2 mM	42.35 ± 2.58^{C}	41.45 ± 2.43^{bBC}	37.40 ± 1.59^{abAB}	37.05 ± 2.40^{AB}	36.25 ± 2.03^{A}
	MEL 3 mM	43.50 ± 2.93^{B}	42.00 ± 3.31^{bAB}	40.10 ± 2.88^{bAB}	38.15 ± 2.46^{AB}	36.35 ± 2.05^{A}
	MEL 4 mM	38.75 ± 2.66^{B}	38.15 ± 2.56^{aB}	37.75 ± 2.17^{abAB}	35.80 ± 2.32^{AB}	34.25 ± 1.76^{A}
	MEL 5 mM	38.45 ± 3.40^{B}	35.80 ± 2.36^{aAB}	35.75 ± 1.96^{aAB}	34.35 ± 2.12^{AB}	33.70 ± 2.34^{A}
Wobble (WOB)	Control	81.82 ± 11.29	65.71 ± 8.05	51.40 ± 3.86	51.83 ± 2.12	52.45 ± 3.39
	MEL 1 mM	47.02 ± 3.04	61.06 ± 3.92	53.76 ± 2.26	59.65 ± 4.62	59.74 ± 6.56
	MEL 2 mM	54.47 ± 1.78	52.40 ± 1.98	51.63 ± 1.72	53.05 ± 2.91	53.35 ± 2.10
	MEL 3 mM	63.51 ± 4.03	57.40 ± 3.38	51.30 ± 1.70	58.43 ± 4.00	51.99 ± 2.47
	MEL 4 mM	54.09 ± 1.92	51.78 ± 1.61	53.08 ± 3.94	53.04 ± 3.76	52.28 ± 2.38
	MEL 5 mM	78.11 ± 10.50	53.36 ± 1.85	55.57 ± 4.61	58.60 ± 7.63	59.21 ± 5.22

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$).

4 Discussions

The results showed that addition of MEL has improved the motility and velocity parameters of mithun semen. Based on the perusal of literature, no reports on inclusion of MEL on mobility and velocity parameters in mithun species and this is the primary report to our the best of knowledge. Earlier workers reported that MEL has significantly higher benefit on refrigerated preservation mammalian sperm and also enhanced the velocity and mobility parameters in the present study (Ashrafi et al., 2011; Ashrafi et al., 2013; Du Plessis et al., 2010) and also on semen quality parameters (Casao et al., 2009; Ashrafi et al., 2011; Hyun-Yong et al., 2006; Ashrafi et al., 2013; Du Plessis et al., 2010; Perumal et al., 2013; Perumal et al., 2015).

The MEL functioned on dose depended method (Casao et al., 2010; Perumal et al., 2013; Perumal et al., 2015) as 3 mM MEL is the most suitable and optimum dosage. Similar observation was reported that MEL had induced boar spermatozoa to a hyperactive state(Martin-Hildago et al., 2011)as the result of an elevated synthesis of ATP; MEL is known to promote mitochondrial complex efficiency and ATP production (Martin et al., 2000). MEL has improved the velocity and mobility parameters in the current study as because of its interaction with second messenger calmodulin in the sperm (Benitez-King & Anton-Tay, 1993) and which inturn stimulate the cytoskeletal structures of sperm leads to higher sperm velocity and motility. Moreover, the MEL is acts as an antioxidant and an antiapoptotic agent in sperm storage medium and it protects the sperm through inhibition of ROS generation, caspase-3 and caspase-9 activities, phosphatidylserine externalization, apoptosis and sperm death (Espino et al., 2011) and through which it protects the sperm and its mitochondrial potential for energy production to progress forward direction.

Improved actions of MEL is due to it increases the ATPase production (Chen et al., 1994), which is the source energy and used by the sperm and activate motility and velocity (Burger et al.,1991). According to Delgadillo et al. (1994) MEL also stimulates cellular influx of Ca^{2+} and enhancing motility. Further, Si (1997) suggested that Ca^{2+} regulate the flagella movement and calmodulin have been identified in the spermatozoa and flagellar (Tash & Means, 1983). According to Ahmad et al. (1996) calmodulin antagonist caused a reduction in VCL and ALH and mitochondrial membrane potential. Moreover, MEL has improved the parameters act on the cAMP (Yung et al., 1995) and stimulates velocity (Lindamann, 1978) and/or acting on secondary messenger (Garbers & Kopf, 1980).

The results of the present study revealed that inclusion of MEL @ 3 mM has improved the keeping quality, mobility and velocity parameters of mithun sperm preserved at 5°C for 30 hrs of incubation. Motility and velocity parameters of the sperm were decreased during the time of storage and remedially maintained above 50% for upto 30 h period of time. In contrarily, decreasing rate in the motility percentage and velocity rate were higher in the ejaculate treated with 4 to 5 mM MEL or without MEL. However, inclusion of 3 mM MEL, the velocity and motility parameters were higher as compared to untreated control group in the present study (Du Plessis et al., 2010). Various effects of MEL at various might be described according to the observation reported by Ashrafi et al. (2011), Shoae & Zamiri (2008), Perumal et al. (2013) and Perumal et al. (2015) revealed the excessive amount of MEL than optimum inturn to higher fluidity of plasma membrane of sperm, creating the sperm are more prone to plasma membrane and acrosomal damages and also inclusion of high dosage leads to deleterious effect on the spermatozoa as because alteration in physiological and physical condition of diluent. But the antioxidant concentration higher than required amount was deleterious and toxic to spermatozoa (Maxwell & Watson, 1996; Perumal et al., 2013; Perumal et al., 2015). However, reduced concentration also altered the sperm parameters and structures. Therefore based on the present study, mobility and velocity parameters were increased maximum upto 3 mM then reduced to 5 mM.

Inclusion of exogenous MEL improved semen quality, motility, acrosomal membrane quality and viability of semen, similar types of results was also reported by Casao et al. (2009) and Ashrafi et al. (2011) and by various researchers in various organism such as bull sperm (Ashrafi et al., 2013), mithun (Perumal et al., 2013; Perumal et al., 2015) and boar sperm (Hyun-Yong et al., 2006). Furthermore, MEL protects plasma membrane, mitochondrial membrane integrity, acrosomal membrane and functional structure of flagella of sperm, cytoskeleton structure as cell protecting effects (Leon et al., 2005).

MEL has also protects and stimulates the functions of antioxidant enzymes like SOD, GSH and CAT (Karbownik & Reiter, 2000), which helps to maintain membrane integrity, membrane transportation process (Alvarez & Storey, 1992) and fertility rate of the sperm cells. Further it reduces the number of free radicals, ROS indirectly, and it also may enhance the production of sperm protecting molecules against oxidative and peroxidative stress. Through these mechanisms, the velocity and motility parameters of sperm were increased significantly by using MEL in the current study.

It was concluded that the possible protective effects of MEL supplementation were it enhanced the mobility and velocity parameters assessed by computer assisted sperm analyser at 3 mM in future, cryopreservation studies is needed to confirm the present research findings.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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SERO MONITORING OF ANTI RABIES VACCINAL ANTIBODIES TO PEP IN ANIMALS BY RFFIT - A CASE STUDY IN KARNATAKA

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ABSTRACT

A dog suspected to be affected with rabies attacked 31 animals in Kesarahatti village of Gangavathi taluk, Koppal District in Karnataka on 14th June 2015. Of these, 26 animals (14 cattle, 5 buffaloes and 7 dogs) were given the first aid followed by post exposure anti rabies vaccine at 1 (15th June 2015), 3, 7, 14 and 28 days interval whereas the remaining 5 animals (2 cattle and 3 buffaloes) reportedly died of suspected (based on clinical manifestation) rabies after 8 days of dog bite episode. These 5 animals were not provided first aid and post exposure prophylactic (PEP) vaccination as their exposure to the rabid dog bite went unnoticed. Further, the serum samples could be collected from only 21 out of 26 vaccinated animals on 28th and 90th day post vaccination. These serum samples were tested by Rapid Fluorescent Focus Inhibition Test (RFFIT) and the titre of neutralizing antibodies ranged from 1-8 IU/ml from the serum collected on 28th day post vaccination indicating protection in all the vaccinated animals. But the titre ranged from 0.5-4 IU/ml in 18 animals and less than 0.5 IU/ml in three animals (2 cows and a dog) on 90th day post vaccination. The protective level of neutralizing antibody titre on day 28 post vaccination and non-development of disease suggests that the PEP vaccination could be effective in livestock avoiding euthanasia and culling of animals.

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1 Introduction

Rabies is a viral zoonotic neuro invasive disease infecting all mammals and is usually fatal. In India, dogs are the major reservoirs of rabies virus and grazing livestock are commonly prone to rabid dog bites. The clinical manifestations are highly variable. The acute, maniacal, furious form is accompanied by hyperesthesia, bellowing, ataxia, charging of animate or inanimate objects, sexual excitement and rarely blindness and seizures (Lahunta, 1983). The paralytic form is rarely manifested in livestock.

The concept of post exposure therapy with tissue culture rabies vaccine is practiced in humans throughout the world. However, there are scanty reports on post exposure prophylactic vaccination trials in canines and other livestock including bovines (Basheer et al., 1997; Hanlon et al., 2002; James et al., 2007; Manickam et al., 2008). Post exposure treatment in animals is still controversial despite the fact that it has been practiced on large scale in certain situations in developed countries (Clark & Wilson, 1996). The PEP treatment has been described for variety of species both experimentally and for field condition (Ramanna et al., 1991; Basheer et al., 1997). The data available regarding the efficacy of PEP and monitoring of anti-rabies vaccinal antibodies in domestic animals is scanty. Keeping this in view and frequent reports of rabies in cattle especially in the rural areas, in the present study, the PEP vaccination was evaluated by assessing anti rabies vaccinal neutralizing antibodies in different animal species using different vaccine brands.

2 Materials and Methods

2.1 Animals

A dog suspected to be affected with rabies attacked 31 animals in Kesarahatti village of Gangavathi taluk, Koppal District in Northern part of Karnataka on 14th June 2015. Of these, 26 animals (14 cattle, 5 buffaloes and 7 dogs) were seen being bitten by the rabies suspected dog whereas the remaining five animals (2 cattle and 3 buffaloes) were not seen being bitten by the suspected rabid dog. Hence only 26 animals were subjected to PEP vaccination. But, remaining five animals which were unvaccinated, died after 8 days of the incidence exhibiting symptoms suggestive of rabies. However, the post mortem brain sample of the rabies suspected dog or the five animals were not available for laboratory confirmation.

2.2 Antirabies vaccination

The twenty six animals bitten by the rabies suspected dog were subjected to the first aid followed by initiation of post exposure anti rabies vaccine at day 1(15th June 2015), 3, 7, 14 and 28 by intra muscular (I/M) route. The Essen regime used for prophylaxis in humans was followed. Only 21 animals could be administered with the complete schedule of 5 shots since 5 animals were sold by the owners before the completion of the regimen. The vaccines used were BHK 21 cell culture inactivated vaccine "Raksharab", inactivated tissue culture vaccine “Anirab H” and inactivated vaccine containing Aluminium phosphate as an adjuvant “Nobivac R” used in dogs. All the animals were observed for 90 days after treatment for development of the clinical symptoms.

2.3 Serum samples

The blood samples were collected on 28^{th} day and 90^{th} day post vaccination from only 21 animals which received vaccine for the complete duration as the remaining 5 animals were sold and not traceable. The serum was separated in aseptic manner and stored at -20 ºC until subjected for test.

2.4 Rapid Fluorescent Focus Inhibition Test (RFFIT)

The titration of rabies neutralizing antibodies was carried out using RFFIT a “gold standard” *in-vitro* test according to Smith et al., 1996 using the BHK 21 cell line. 100 $TCID_{50}$ of rabies virus PV 3462 (Dr. Larghi’s strain) provided by Pasteur Institute, Coonoor, Tamil Nadu in 96 well microtitre plate. The WHO international standard for rabies Immunoglobulin (NIBSC, potters Bar, UK) was used as a positive control.

In brief, two fold serially diluted (1:2 to 1:16) heat inactiviated test serum samples were mixed with 100 $TCID_{50}$ Dr. Larghi’s strain of rabies virus and incubated at 37 ºC for 90 minutes with 5 %CO_2 for neutralization. About 60 µl of 3 to 4 days old BHK cells (25,000 to 30,000 cells/well) were added to all the wells. Standard WHO reference serum and negative serum was included in each test. A virus and cell controls were maintained. The microtitre plate was incubated at 37 ºC in an atmosphere of 5 %CO_2 for 48 hours. After incubation, the assay plate was observed under microscope for cell confluence.

The medium was decanted from the plate without disturbing the monolayer and 100µl 70% ice cold acetone was added and the plate incubated for 30 minutes at - 20ºC and thereafter acetone was decanted and allowed the plate to dry. Further, 50µl of Rabies anti- nucleocapsid conjugate (Light Diagnostics, Rabies DFA III Cat # 6500) at working dilution (1:100) was added to cover the entire monolayer and the plate incubated at 37 ºC with 5 %CO_2 for 45 minutes. After incubation, the plate was washed with sterile Phosphate Buffer Saline twice. The plate was observed under fluorescent microscope at 20x objective. The antibody titers of serum were expressed in International Unit (IU) per milliliter (IU/mL) by comparing results obtained with those of the positive standard. A titre of minimum 0.5 IU/ml was considered protective as per WHO.

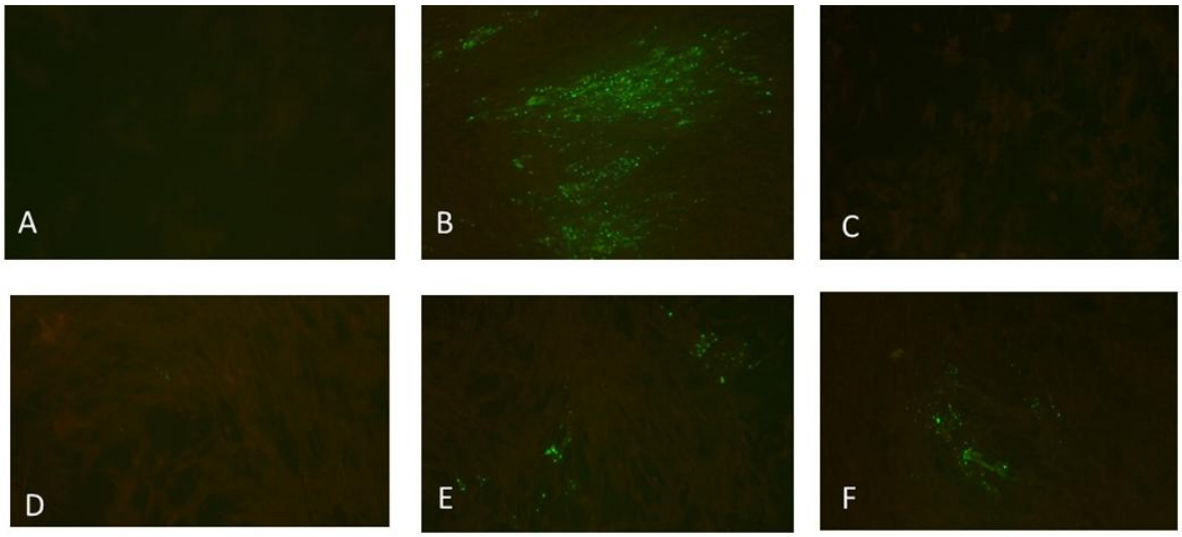

Figure 1 RIFFIT analysis in microscope at 100X magnification. A. Cell control; B : Virus control showing fluorescing foci.C: 1 in 2 dilution of serum sample (0.5 IU) without any fluorescing foci, D, E, and F are 1 in 4, 8 and 16 dilution of serum sample (0.5 IU) showing fluorescing foci.

3 Results and Discussion

In this episode of a suspected rabid dog bite, all the animals which received PEP were under observation for 90 days. None of the animals showed clinical signs during the observation period although most of them had received a bite on their face. Five of the animals (2 cattle and 3 buffaloes) which had not received the PEP died after 8 days of dog bite, exhibiting symptoms suggestive of rabies. According to Starr (2001), there is a latent period of three to four days before multiplication and attachment to nerve endings occur. Whether there is a short or long incubation period depends on a number of factors: the length of temporary arrest of virus multiplication, either at the site of deposition or at some location in the nerve system; the number of viral particles deposited in the tissues, and the virulence of the particular strain of virus. The street virus rabies is characterised by a variable incubation period. In the present study, RFFIT was employed to estimate the anti-rabies neutralizing vaccinal antibody titres and a minimum of 0.5 iu was considered protective (Figure 1). All the post vaccinal sera collected showed antibody titer on day 28 and lower titers at three months. Further, all the three vaccine brands used by employing the Essen regime were found to be protective in all the vaccinated animals similar to the observation of Basheer et al. (1997). A titre of >0.5 IU (1-8 IU) was evident in case of all the vaccinated animals on the 28th dpv. However, the neutralizing anitbody response was seen to be protective (in the range of 0.5 – 4 IU) in case of 18 animals and was <5 IU in the remaining 3 animals on the 90th dpv (Table1).

This finding indicated the elevation of antibody response on day 28th and waning of the same on the day 90th. Similar observations of peak antibody titre during 3-6 weeks post vaccination and waning of antibody titre were made earlier (Aubert, 1992; Oliveira et al., 2000; Manickam et al., 2008; Minke et al., 2009). Although reduction in the antibody response on the day 90 was expected, it's reduction below 0.5 IU in case of 3 animals was unexpected. Although various factors including the breed, size, age, maintenance of cold chain, regimen employed could influence the titre, in this study it is difficult to attribute any specific reason to explain such reduction in the titre <0.5 IU.

Nevertheless, the initial elevation of antibody response >0.5 IU in case of all the vaccinated animals is important to neutralize the virus as observed. Some studies conducted earlier (Cho & Lawson, 1989; Clark & Wilson, 1996) included a booster vaccination on the day 90. However, the study supports the findings of Shayam et al. (2006), Hoque et al. (2006) and Wilson & Clark (2001) revealing a protective titer after 5 vaccinations *i.e.*, day 0, 3, 7, 14 and 28 days. Although on 90 dpv, the virus neutralizing titers irrespective of the vaccine used appeared to be waning, it is likely that the immunity thus stimulated would last for a much longer period. Manickam et al. (2008) challenged dogs intramuscularly with virulent rabies virus brain suspension and injected intramuscularly with either Nobivac Rabies (Intervet), Rabisin (Merial) or placebo on multiple occasions (3 or 5-times) over the next 28 days. They recorded the serum antibody production after 3 days of post vaccination with maximum antibody titre seen on 28th dpv in dogs. In their study, the neutralizing antibodies had declined by day 90. They also compared response of antibody production using 2 different cell culture rabies vaccines in Indian street dogs and concluded that both vaccines were found to be safe and effective similar to the observations of our study.

Table 1 Details of neutralizing antibody response in vaccinated cattle, Buffaloes and Dogs.

Sl. No.	Animal	Age	Sex	Vaccine used	Place of bite	Antibody titer of samples collected on day 28 (IU)	Antibody titer of samples collected on day 90 (IU)
1	Cow (HF CB)	6 Month	Male	Raksharab	Face	8	1
2	Cow (HF CB)	5 yr	Female	Anirab	Leg	1	1
3	Cow (HF CB)	8 month	Female	Raksharab	Nose	8	2
4	Cow (HF CB)	6 month	Female	Raksharab	Face	4	< 0.5
5	Cow (HF CB)	6 yr	Female	Raksharab		1	2
6	Cow (JR CB)	6 yr	Female	Raksharab		2	4
7	Cow (HF CB)	6 month	Female	Raksharab		4	< 0.5
8	Cow (HF CB)	6 yr	Female	Raksharab		2	4
9	Bullock	6 yr	Male	Anirab	Face	1	0.5
10	J Cow (JR CB)	7 yr	Female	Raksharab	Face	2	0.5
11	Cow (JR CB)	6 yr	Female	Raksharab	Face	1	4
12	Cow (local)	5 yr	Female	Raksharab	Leg	1	2
13	Buffalo	8 month	Male	Raksharab	Face	4	1
14	Buffalo	6 yr	Female	Raksharab	Nostrils, eye and head	1	2
15	Buffalo	6 month	Female	Raksharab		4	2
16	Buffalo	6 month	Female	Raksharab	Face	2	2
17	Dog (GSD)	9 month	Male	Raksharab	Legs	2	4
18	Dog (Street)	-	-	Anirab	Face	2	< 0.5
19	Dog (Street)	-	Male	Anirab		4	4
20	Dog (Street)	-	Male	Raksharab		2	2
21	Dog (Dachshund)	1.5 yr	Male	Nobivac R	Face	4	2

Conclusion

In conclusion, the PEP could be effective in livestock, although there was reduction in the neutralizing antibody titre below protective level of 0.5 IU in 3 out of 21 vaccinated animals at the end of 90 dpv, probably the elevation of antibody level above the protective level on day 28th dpv must have conferred protection. Despite these encouraging observations, the present limited field based study may not necessarily suggest the replacement of quarantine by PEP unless more such field based investigations are done especially with a view to ensure the elevated antibody response (>0.5 IU) beyond 90 days.

Furthermore, simultaneous administration of rabies immunoglobulin (RIG) at the local site of bite (subject to cost effectiveness) in case of category 3 bites along with PEP vaccination shall be explored in animals as is being done in human beings. Such data could enable undertaking routine PEP vaccination in livestock exposed to rabies and thereby avoid euthanasia or prolonged quarantine.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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EXPRESSION AND LOCALIZATION OF BONE MORPHOGENETIC PROTEIN6 (BMP6) IN THE CORPUS LUTEUM DURING DIFFERENT STAGES OF ESTROUS CYCLE IN THE BUFFALO (*Bubalus bubalis*)

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ABSTRACT

Emerging evidence suggests that Bone Morphogenetic Proteins (BMPs), which belong to the Transforming Growth Factor-β (TGF-β) super-family, are known to be involved in the follicular growth and steroid production in different species. This study describes BMP6 bearing in corpus luteum over various stages of estrous cycle. The bubaline CL was classified into four stages according to the morphology and progesterone (P_4) concentration. The real time PCR and immunoblot studies revealed that BMP6 was significantly ($P<0.05$) unregulated during the mid stage of CL that was consistent with immunohistochemical localization in the luteal cells. The transcriptional and translational expressions of BMP6 in the early and late CL were comparable and significantly ($P<0.05$) lower than that of mid CL. In conclusion, BMP6 expression is dependent on the stage of CL in the buffalo.

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1 Introduction

Livestock production constitutes a very important element of the agricultural economy of developing nations. In India, milk, meat and draft purposes is fulfilled by water buffalos (*Bubalus bubalis*) and act as an important livestock for rural community. Buffalo milk shares 62% of total national milk production in India (DAHDF, 2012). According to FAOSTAT (2012), there are 194.2 million heads of buffalo in the world, of which 115.4 million is in India (59.4% of total world population). High milk yield with more fat content, good feed conversion efficiencies, comparatively higher disease resistant than the bovines are the peculiarities of the buffaloes (Kumar et al., 2012). Owing to the versatility of buffaloes, they are called 'black gold' by the farmers (Bilal et al., 2006). However, the buffalo is considered to be a poor breeder due to high incidence of delayed puberty, silent estrus, summer anestrus and long postpartum interval (Barile, 2005; Madan & Prakash, 2007). Anomalies in the angiogenesis and vascularization of the corpus luteum (CL) may result in premature luteal regression, decreased progesterone (P_4) production and unexplained infertility (Madan & Prakash, 2007). Hence, there is a need to study the role of intraluteal autocrine and /or paracrine factors with respect to luteogenesis, luteostasis and luteolysis.

It is known that the luteal function is under the negative feedback control of hypothalamo-hypophyseal ovarian axis. But, it is well established that growth factors produced locally had crucial permissive and modulatory function in growth of follicles, ovulation, dominance and luteal function (Fortune et al., 1988; Hyashi et al., 2003; Berisha & Schams, 2005). These local factors include Transforming growth factor-β (TGF-β),Vascular endothelial growth factor (VEGF), Insulin-like growth factor (IGF), Fibroblast growth factor (FGF), Epidermal Growth Factor (EGF) and angiopoietin etc. (Ferrara et al., 2003; Kaczmarek et al., 2005; Bramley et al., 2005; Otrock et al., 2007). Among the ovarian factors, members of the TGF-β super family have a biological role in the folliculogenesis in the cow (Knight & Glister, 2006; Glister et al., 2010).

Bone morphogenetic proteins (BMPs) are categorized under the TGF-β super family. BMPs are a group of bioactive proteins that were originally isolated from the extract of bone matrix of by Urist in 1965. To date, over 20 BMPs have been identified and shown to be involved in the regulation of cell proliferation, survival, differentiation and apoptosis, chondrogenesis, osteogenesis and embryogenesis. BMPs exhibit their actions by stimulating the membrane attached threonine/serine kinase receptors. The BMPs transduce the signals *via* the classical BMPs–Receptor–Smads signal pathway (Nohe et al., 2004). Recently, BMPs have attracted much attention in the field of ovarian physiology. It is known that the BMP receptor mRNAs are present in the ovary, with the strongest expression in the granulosa cell (GC) and in the oocyte, which is consistent with the BMP's action observed on the GCs (Shimasaki et al., 1999; Wilson et al., 2001).

The BMPs function as luteinization inhibitors by suppressing luteinizing hormone (LH) receptor expression in the GCs (Shimasaki et al., 2004). Additionally, the BMP system was shown to play a crucial role in folliculogenesis in humans (Shi et al., 2009; Shi et al., 2010; Shi et al., 2011). Of the BMPs, BMP6 is highly expressed in the theca cell layer in the ovarian follicles and CL of rat (Shimasaki et al., 2004) and cow (Glister et al., 2010). Until now, no published information is available regarding BMP6 expression and localization in various stages of development of CL in buffalos. Hence, the present study was focused on the mRNA and protein expression along with the immunohistochemical localisation of BMP6 in the CL during different stages of the estrous cycle in the buffalo.

2 Materials and Methods

2.1 Collection of corpus luteum

Buffalo cow genetalias which are apparently normal were collected from the local slaughter houses and transported with the help of ice to the laboratory. The stage of estrous cycle was elucidated in virtue of the macroscopic findings of the ovaries and uterus (Sarkar et al., 2010). The CL was categorized into early luteal, mid luteal, late luteal and regressed stages with 10 CL at each stage. Further, luteal sample was frozen in liquid nitrogen and stored at -80^{o}C until RNA and protein extraction (Kumar et al., 2012).

2.2 Follicles collection

Ten follicles which seems healthy, transparent, highly vascular and with more than 14 mm diameter were utilized in this study. Preovulatory follicles were selected based on regressing CL and mucus secretion in uterus. Follicles were removed from ovaries and the theca externa were removed from the follicles with the help of forceps and stereomicroscope (Sarkar et al., 2010). The aspirated follicular fluid was stored at -20^{o}C for estimation of progesterone (P_4). Normal follicles are having relatively constant levels of P_4, so follicles having P_4 concentration less than 100 ng/mL were utilized in the present study (Kumar et al., 2012). Follicles were frozen with the help of liquid nitrogen and stored at -80^{o}C.

2.3 Determination of Hormone

As per the instruction of manufacturer (Immunotech, Czech Republic) using $P_4$125I RIA kit (IM1188) the P_4 concentration in FF were estimated. The FF was diluted with phosphate-buffered saline (PBS) with the dilution ratio of 1:5. 0.05 to 50 ng/mL was the measurable range. The inter and intra assay coefficients of variation was 7.2 and 6.5%, respectively.

Table 1 Target gene, primer sequences and amplicon length for qRT-PCR used in the study.

Gene	Sequence of nucleotide (5'-3')	Efficiency (%)	Amplicon length (bp)	EMBL accession No. or reference
BMP6	Forward:GGCCCCGTTAACTCGACTGTGACAA Reverse: TTGAGGACGCCGAACAAAACAGGA	101.2	108	XM_600972.2
RPS15A	Forward: AGGGCTGGGAAAATTGTTGTGAA Reverse: TGAGGGGATGGGAGCAGGTTAT	104.8	125	Mishra et al.,2015

BMP, Bone morphogenetic protein; **EMBL,** European molecular biology laboratory; RPS15A-Ribosomal protein 15A; qRT-PCR, Quantitative real time polymerise reaction.

2.4 Primers

Details of primers used are presented in Table 1. BMP6 primer was designed by employing Fast PCR software (6.5.63 version).

2.5 Quantitative RT-PCR

RNA was extracted from follicles and different stages of CL using TRIzol method (Invitrogen®). The integrity of RNA was assessed in 1% agarose gel with 1X tris-borate-EDTA (TBE) buffer, and purity and concentration of RNA was estimated in Nanodrop (Bio-Rad Laboratories, Hercules, CA). Constant amount of total RNA (1 µg) was reverse transcribed using cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA) and oligo-dT18 primer at 42^{o}C for 90 min. The resulting complementary DNAs were used in qPCR. The pre-ovulatory follicle (PF) was used as calibrator for obtaining relative mRNA expression.

The qPCR for each complementary DNA and the housekeeping gene RPS15A was performed in duplicate using SsoFast Eva GreenSupermix kit (Bio-Rad) in a Biorad CFX manager Real-Time qPCR System instrument as per manufacturers' instructions. The efficiency for the primer of different factors has been given in Table 1.

0.5 µL of cDNA as PCR template was added to 0.25 µL of forward and reverse primer (0.2 mM) each, and 5 µL of SsoFast Eva Green Supermix and final volume of 10 µL was adjusted with nuclease free water (NFW) and subjected to qPCR. The following general qPCR protocol was used for BMP6: enzyme activation for 30 sec at 95^{o}C, 40 cycles of a 3-segmented amplification and quantification program (denaturation for 5 sec at 95^{o}C, annealing for 10 sec at the primer-specific temperature (58^{o}C for BMP6, 60^{o}C for RPS15A) and elongation for 15 sec at 72^{o}C), a melting step by slow heating from 61 to 95°C with a rate of 0.58^{o}C/sec and continuous fluorescence measurement and a final cooling down to 4^{o}C. After the run ended, cycle threshold values and amplification plot for all determined factors were acquired using the "EVA green (with dissociation curve)" method of the real-time machine (Biorad CFX manager Real-Time qPCR^{TM} software) qPCR efficiencies were determined by amplification of a standardized dilution series and slopes were obtained. The specificity of the product was checked in gel electrophoresis. Negative control was run by adding all the components except template to rule out the formation of primer dimer.

2.6 Antibodies

Immunoblotting and immunohistochemistry were performed using goat polyclonal GAPDH (sc-48166; Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit polyclonal BMP6 (Catalog# YPA1178; Chongqing Biospes Co., Ltd), goat anti-rabbit IgG-HRP (sc-2004, catalogue no. D2114), mouse anti-goat IgG-HRP (sc-2354, catalogue no. B1815) and goat anti rabbit IgG-FITC (sc-2012, catalogue no. 1010).

2.7 Western blot

To obtain total proteins, liquid nitrogen triturated luteal tissues of different stages were suspended in RIPA lysis (Ameresco, UK) buffer and Halt protease inhibitor cocktail (Thermo Scientific,USA), homogenized, and centrifuged at 12,000g. Total protein concentration was estimated using Bradford protein assay and supernatant was diluted in sodium dodecyl sulfate (SDS) buffer (final concentration to 60 mM Tris, 2% SDS, pH 6.8, 10% glycerol and 100 mM dithiothreitol), followed by boiling for 5 min.

The protein samples (100 mg from each group of CL) were subjected to SDS 10% polyacrylamide gel electrophoresis, electro transferred onto polyvinylidene difluoride membrane, and blocked with 5% bovine serum albumin (BSA) before incubation with primary antibody namely, BMP6 at a 1:200 dilution and polyclonal GAPDH at a 1:500 dilution for overnight at 4^{o}C. After incubation, the membrane was washed thrice with PBS-T (PBS 0.01% Tween 20) for 5 min each and the respective secondary antibody conjugated with horseradish peroxidase was added and incubated at 37^{o}C for 1 h. After washing 3 to 4 times in PBS-Tween 20 solution, the positive signals were detected by incubating the membrane using 0.06% 3,30-diaminobenzidine tetrahydrochloride (Genei) in 1XPBS (pH 7.4) containing 0.06% H_2O_2 for 10 to 15 min. Under white light, the bands were visualized and captured with digital camera.

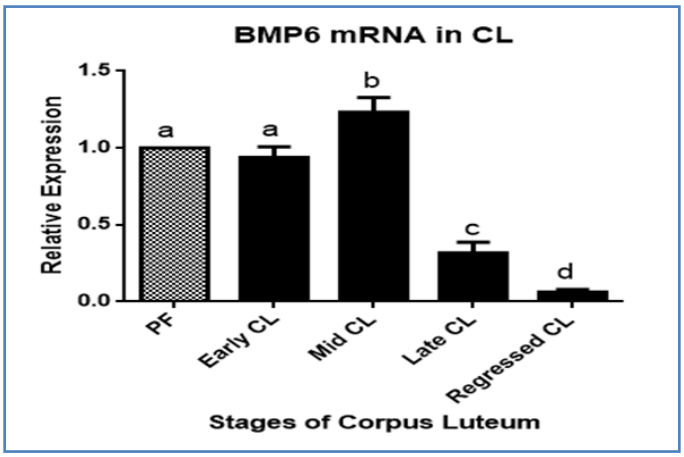

Figure 1 Expression of BMP6 at different stages of CL development in the buffalo (n = 10/group). (early CL, days 1–4, mid CL, days 5–10, late CL, days 11–16, and regressed CL, days >17 of estrous cycle). The PF served as calibrator group to calculate the fold change. RPS15A was used as reference gene to calculate ΔCt (delta C_t). One-way ANOVA was done to find the between group difference and Tukey honest significant difference test was done to find the pair-wise mean difference. Minimum level of significance was set at 95%. Each bar represents Mean ± SEM. Bars with different superscripts denote significant difference (P<0.05). Abbreviations: CL, Corpus luteum; mRNA, Messenger RNA; BMP, Bone morphogenetic protein; PF, Preovulatory follicle; RPS15A, Ribosomal protein 15A; ANOVA, Analysis of variance.

2.8 Immunohistochemistry

Freshly collected luteal tissues were fixed with 10% neutral buffer formalin, dehydrated through a series of graded alcohols, paraffin-embedded, serial-sectioned (5 µm), mounted on 3-Aminopropyl triethoxysilane (Thermo Scientific) coated slides and dried at 37°C overnight. Deparaffinization in xylene and rehydration in a series of graded alcohols at room temperature was done. Antigen retrieval was done in 10 mM sodium citrate buffer (pH 6.0, 0.05% Tween- 20) and rinsing thrice in PBS 5 min each. Blocking of non specific sites was done with 5% BSA for 2 h at 37°C. Subsequently, sections were probed with BMP6 antibody at 1:200 dilutions. Primary antibody was detected by fluorescent conjugated goat anti rabbit IgG-FITC secondary antibody at 1:400 dilution. Rinsing of slides were done thrice with the help of PBS and DAPI was used to stain nuclei. Without the addition of primary antibody, the control slides were stained with addition of isotype IgG. The slides were mounted with antifade solution (MP Biomedicals) and images were taken in AxioObserver.Z1 microscope (Germany).

2.9 Statistical analyses

All the experimental data were expressed as Mean ± SEM. The statistical significance of difference in mRNA expression of the examined factor across different stages of estrous cycle and the expression of protein was assessed using the software SPSS22 (IBM Corporation) by one-way analysis of variance followed by Tukey honest significant difference (HSD). Differences were considered significant if $P < 0.05$.

3 Results

3.1 Expression of mRNA for BMP6 in the corpus luteum

BMP6 was found to be expressed in a regulated manner with stage specific differences in the expression pattern in different stages of CL development in buffalo. The relative mRNA expression of BMP6 was significantly (P<0.05) upregulated during the mid luteal stage compared with the early and regressing stages of CL (Figure. 1). The expression of BMP6 in the early and late CL was comparable and significantly lower than the mid CL.

3.2. Western blot analysis

The BMP6 and GAPDH proteins were visualized on western blot analysis as bands of molecular weight approximately 54.4 kDa and approximately 37.5 kDa, respectively (Figure. 2A). The highest protein expression was identified during mid and late luteal stage which is correlated with mRNA expression.

3.3. Immunohistochemistry

The localization of BMP6 protein was conspicuous in various cell types in different stages of CL sections. The immunoreactivity was exclusively found in the cytoplasm of luteal cells and was greater during mid and late stages for BMP6. The negative controls did not show any specific immunoreactivity.

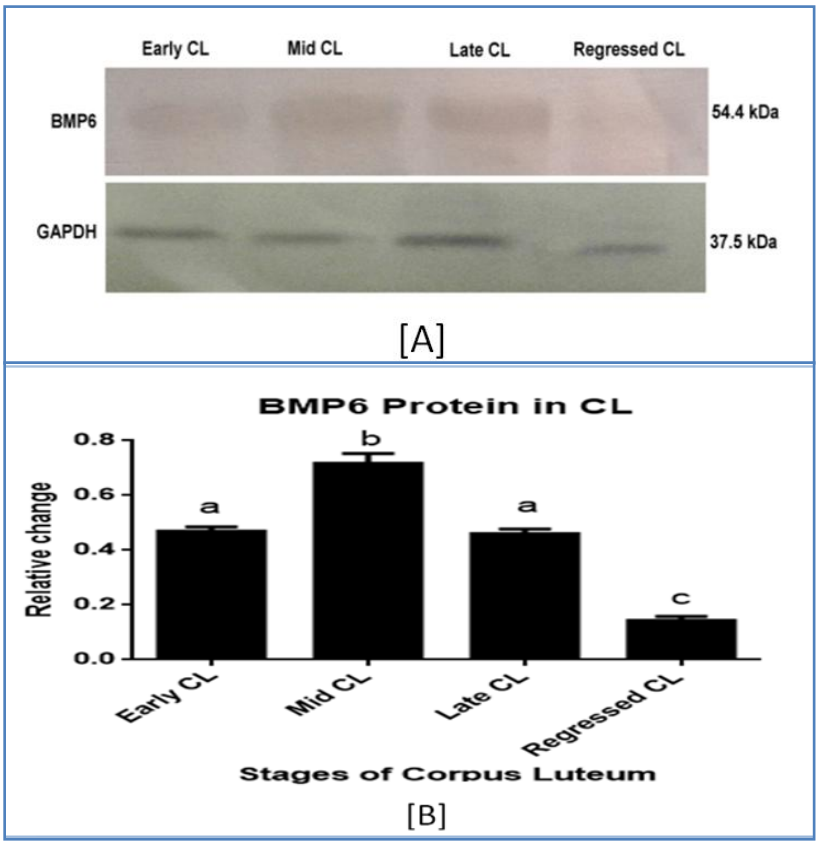

Figure 2 Demonstration of BMP6 protein by immunoblotting at different stages of CL development in the buffalo. Luteal protein was loaded at 100 mg/well, resolved in 12.5% SDS-PAGE and electrotransferred to the PVDF membrane. Primary antibody was used at 1:200 while secondary goat antirabbit antibody was used at 1:5000**.**

[A) Representative blot of BMP6 and GAPDH. The relative molecular weight of each BMP is indicated on the right end of each blot. GAPDH was used as reference protein **B)** Relative expression of BMP6 protein (Band Densitometric analysis of the immunoblot) was done using image J software (n=6/group). One-way ANOVA was done to find the between group difference and Tukeyhonest significant difference test was done to find the pair-wise mean difference. Minimum level of significance was set at 95%. Each bar represents Mean ± SEM. Bars with different superscripts denote significant difference ($P<0.05$). Abbreviations: BMP, Bone morphogenetic protein; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; CL, Corpus luteum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase ; PVDF, Polyvinylidene fluoride; ANOVA, Analysis of variance.]

4. Discussion

In the ovary, investigations on BMPs are mainly studied in the follicle of rat (Erickson & Shimasaki, 2003), cow (Glister et al., 2010) and ewe (Juengel et al., 2006). Except a solitary report by Erickson & Shimasaki (2003) on rat, no study could be found on BMPs in the CL. To the best of our knowledge, this is the first study on BMP6 in the CL of riverine buffalo. The mid CL showed a significantly increased expression of BMP6 transcripts (Figure. 1), which is consistent with the results of western blot (Figure. 2A and B) and immunofluorescence (Figure. 3).Though BMP6 mRNA was highly expressed in the theca cells and the oocytes of rat (Erickson & Shimasaki, 2003) and cow (Glister et al., 2004), it was only expressed in oocytes in the follicle of ewe (Juengel et al., 2006). In bovine GCs, BMP6 upregulated basal and IGF stimulated Estrogen (E_2) production (Glister et al., 2004). However, BMP6 suppressed P_4 production in the GC of rat (Otsuka et al., 2001a). In conclusion, BMP6 expression depends on the stage of CL development in the buffalo. Further studies are required to see its expression in the follicle and functional studies using follicular cell culture.

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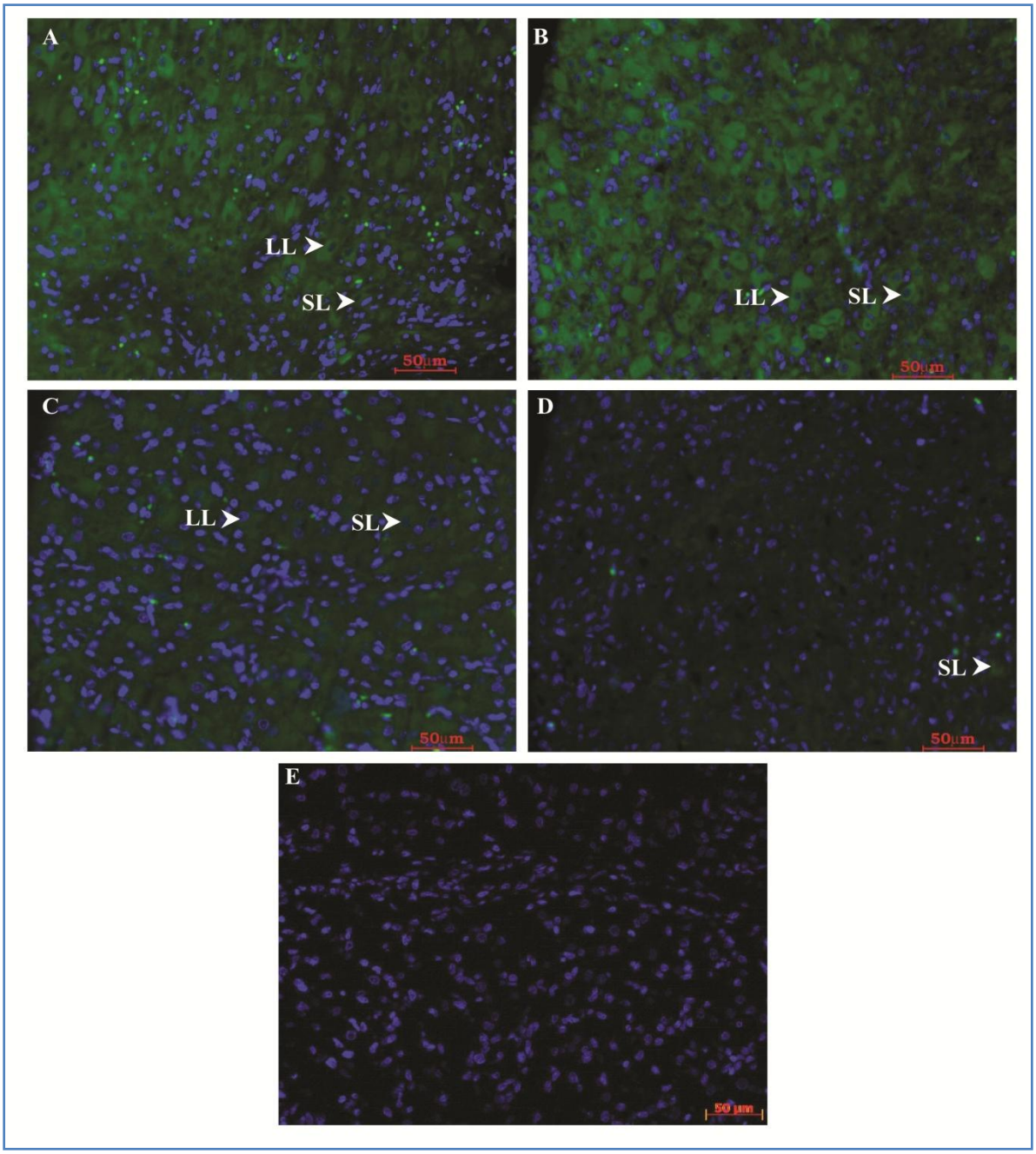

Figure 3 Fluorescent immunohistochemical localization of BMP6 in the CL of buffalo. Briefly, antigen retrieval was done using sodium citrate buffer method on 5 µm thick paraffin embedded sections of CL. BSA 5% was used to minimize the non-specific binding. Primary BMP6 antibody was used at 1:200 while the FITC was used at 1:400. Nucleus was counterstained with DAPI. Green filter was used while examination of the stained sections under the fluorescent microscope (Carl Zeiss Micro Imaging GmbH). Representative images from A through D indicate early, mid, late and regressed stages of CL. No primary antibody was used in the negative control (E). BMP6 was localized predominantly in the cytoplasm of large luteal cells of early and mid CL (A and B) while weak immunoreactivity was seen on late and regressed stages (C and D). Scale bar =50 µm. Abbreviation: LL, large luteal cell; SL, small luteal cell. BSA, Bovine serum albumin; FITC, Fluorescein isothiocyanate, DAPI, 4',6-diamidino-2- phenylindoledihydrochloride.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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EFFECT OF UNILATERAL CRYPTORCHIDISM ON MOBILITY AND VELOCITY PARAMETERS OF SPERM IN MITHUN (*Bos frontalis*) SEMEN

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KEYWORDS

Mithun

Cryptorchid

Computer assisted sperm analysis

Motility

Velocity parameters

ABSTRACT

The present study was conducted to assess the motility and velocity parameters of sperm of normal intact and unilateral cryptorchid mithun bull by computer assisted sperm analyzer (CASA). Twenty ejaculates were collected from cryptorchid (n=10) and normal intact (n=10) bull. The different parameters assessed through CASA were static sperms (SM), Total Motility (TM), forward progressive motility (FPM), Average Path velocity (VAP), Average beat/cross frequency (BCF), Straightness (STR), Average straight line velocity (VSL), Average curvilinear velocity (VCL), Average amplitude of lateral head displacement (ALH), Wobble (WOB), Linearity (LIN), Elongation (EL). The result showed that these CASA parameters differed significantly ($p<0.05$) between the intact and cryptorchid mithun bull and intact mithun bull has significantly ($p<0.05$) higher value than cryptorchid mithun bulls. It was concluded that most of the CASA parameters were significantly lower in cryptorchid bulls than intact bull and however, due to the genetic hereditary nature of cryptorchid, these bulls should not be used for breeding purposes.

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1 Introduction

Cryptorchidism is referred to an abnormal retention of undescended testicle, which may be retained within the abdominal cavity or inguinal canal. Generally, cryptorchidism is detected at time of birth or thereafter shortly. Cryptorchidism may be unilateral or bilateral and unilateral cryptorchidism is more common in all the species. Incidence of cryptorchidism in bovine species is very low as compared to other domestic animals species (about 0.1%) and this defect is hereditary in nature. Among the cryptorchidism, incidence of unilateral was represented as 78, 90, 45-70, 81-93, 66-89, 59, 100 and 62%, respectively for different species such as feline, bovine, canine, equine, humans, porcine, rabbits and ovine, respectively (Amann & Veeramachaneni, 2007). Location of undescended /retention testis also varied depending upon species of the animals (Amann & Veeramachaneni, 2007). In general, cryptorchid testes are hypoplastic and atrophied and do not produce spermatozoa/ aspermatogenesis and bilateral cryptorchids are sterile in nature (Kawakami et al., 1995). But unilateral cryptorchids may be fertile if the descended contralateral testis is functional (Kaki & Sofikitis, 1999). Percentage of infertility in unilateral cryptorchidism differed between 10 (Tellaloglus et al., 1994) to 100 % (Patkowski et al., 1992), but in bilateral, it is 100% as both testes are not functional. Studies were done on different species such as bovine for the harmful effects of cryptorchidism on seminal parameters (Marcus et al., 1997), canine (Kawakami et al., 1995), porcine (Pinart et al., 1999), equine (Ras et al., 2010), ovine (Smith et al., 2012), caprine (Igbokwe et al., 2013) and human (Mieusset et al., 1995).

Subjective analysis of semen is carried out by traditional conventional method like assessment of morphology, viability, concentration and motility by bright field phase contrast microscope in frozen semen bank and andrological laboratory (Iguer-Ouada et al., 2001). The evaluation of semen quality parameters such as sperm head behavior, swimming pattern, motility, velocity etc., may benefit in better understanding of the probable and possible function of sperm, semen quality and selection of suitable semen and bulls for ultralow temperature preservation in the mountain animal species. Various models/methods are available to estimate sperm motility and velocity parameters such as time-exposure or multiple exposure photomicrography to the very sophisticated and accurate analyser like computer-assisted sperm analysis (CASA) techniques (Mortimer, 1997). Sophisticated Computerized method of semen analysis supports accurate, reliable, objective assessment, repeatable results in various semen quality parameters, sperm concentration, total and progressive forward motility and various velocity and motility parameters based on the measurement of single and individual sperm cells (Verstegen et al., 2002).

Similarly, recent finding supported that CASA system not only estimate the proportion of motile spermatozoa but also assess other sperm motion and velocity parameters derived from individual spermatozoa. Further, it has higher predictive power on sperm fertility potential of sperm and semen (Mortimer, 1994). In addition to the use of sophisticated computerized techniques to prognoses the semen fertility, CASA also provides a useful tool to study the effects of different *in vitro* and *in vivo* protocols on sperm velocity and motility as well as the means to study the phenomenon of sperm hyper-activation. Bull fertility was positively correlated with several spermatozoa functional kinematic parametric indexes such as TM, LIN, VSL, PFM, ALH and VCL (Farrell et al., 1996; Perumal et al., 2011). Perusal of literatures showed no reports on velocity and motility parameters in semen mithun suffered cryptorchidism. Hence, the objective of this study was to assess the effect of unilateral cryptorchidism on motility and velocity parameters of mithun semen with computer assisted sperm analyser to pursuit future sperm preservation protocols.

2 Materials and Methods

A mono-cryptorchid and a normal apparently healthy bull of same age with good body condition (score 5-6) maintained under uniform housing, feeding, lighting and managemental conditions at the NRC on Mithun, Nagaland, India were selected for the present study. Assessment and confirmation by several examinations and evaluation like palpation of testes, per rectal examination and visual for cryptorchidism condition were carried out. The semen ejaculates were collected from the mithuns affected and unaffected through transrectal manual massage method two times in a week (Palmer et al., 2004). The necessary experimental protocols performed was whereby carried out as per the regulations implemented by the Institutional Animal Care and Use Committee.

Using the Hamilton Thorne Sperm Analyzer the motility and velocity parameters of sperm were assessed (HTM-IVOS, Version 10.8, MA, USA). The sophisticated computer system contains of a computer analyzing and saving the data, phase-contrast microscope, mini-therm heating stage, camera and image digitizer. The software settings were prepared as follows. Chamber type: Leja 4, fields acquired: 10, temperature of analysis (ºC): 37.0, frame rate (Hz):60, minimum static contrast: 35, number of frames: 30, minimum cell size (pixels): 5, VAP cut – off (µm/s):30, STR (%): 70, Prog.min VAP (µm/s):50, cell intensity: 80, VSL cut-off (µm/s): 15and magnification: 1.89.

Immediately, after semen collection, the sperm concentration was first measured with a phase-contrast microscope (Nikon, Eclipse 80i; 400× magnification). Semen (25 µL) of was diluted with 50-100 µL of Tris (formulated for bull semen) and 5 µL of this extended semen was pipetted into a pre-warmed (37°C) dual chamber disposable Leja slide and was allowed to settle on the mini-therm thermo stage (38°C) just before the assessment of motility and velocity parameters.

Table 1 spermatozoa motility and velocity parameters of cryptorchid mithun bull.

CASA motility and velocity Parameters	Normal intact mithun bull semen (n=25)	Unilateral cryptorchid mithun bull semen (n=25)
Forward Progressive motility (%)	86.00 ± 2.42^{b}	60.90 ± 3.19^{a}
Total Motility (%)	40.80 ± 2.50^{b}	23.50 ± 1.89^{a}
Curvilinear Velocity (VCL) (µm/sec)	260.38 ± 8.43^{b}	190.89 ± 5.73^{a}
Straight line Velocity (VSL) (µm/sec)	102.90 ± 5.34^{b}	68.74 ± 3.21^{a}
Average path Velocity (VAP) (µm/sec)	144.31 ± 6.37^{b}	98.74 ± 3.86^{a}
Linearity (LIN) (%)	49.30 ± 1.84^{b}	36.60 ± 1.61^{a}
Straightness (STR) (%)	78.90 ± 2.04^{b}	67.00 ± 1.76^{a}
Wobble (WOB) (%)	55.22 ± 1.42^{b}	51.93 ± 1.58^{a}
Amplitude of Lateral Head displacement (ALH) (µm)	10.01 ± 1.39^{b}	4.90 ± 1.13^{a}
Beat/Cross Frequency (BCF) (Hz)	27.55 ± 1.84^{b}	21.35 ± 1.96^{a}

Figures with same superscript (a, b) do not differ significantly ($p<0.05$) in rows

Assessment for the following velocity and mobility parameters such as percentage of VCL (µm/sec), BCF (Hz), LIN (%), STR (%), slowVSL (µm/sec), VAP (µm/sec), ALH (µm), WOB (%), velocities of static, medium & rapid, FPM and TM were done through CASA. A Minimum of 200 spermatozoa were assessed from at least different two drops of individual sample from each semen ejaculates. The objects were incorrectly observed as spermatozoa were manually deleted and final assessment was done for each semen sample.

The results in the present study were statistically analysed and presented as the mean ± S.E.M. With the help of student't' test using the SPSS/PC computer program the significant differences between the ejaculates of normal and cryptorchid mithun were estimated (version 15.0; SPSS, Chicago, IL). Values of $p<0.05$ was found for statistically significant differences. With the help of correlation coefficient the correlation between the motility and velocity parameters were established.

3 Results and Discussion

The percent of total motility and forward progressive motility were significantly ($p<0.05$) higher in ejaculates of normal non-cryptorchid than in cryptorchid mithun bulls. Similarly, velocity parameters were significantly ($p<0.05$) higher in normal non-cryptorchid than in cryptorchid mithun bulls (Table 1). Thus, it may enhance the quality of semen by cryopreserving effectively in artificial breeding procedure.

There was no report on motility and velocity parameters of CASA on cryptorchid mithun bull semen and to the best of our knowledge this is the first report of the measurement of motility and velocity parameters by the sophisticated sperm analyser. It is subjective and difficult for determining the sperm motility using the traditional conventional simple microscopical models/ methods. Reports have been made, wherein the estimation of velocity motility parameters of the same ejaculates had shown high variations (Mortimer et al. 1986). Computer sperm analysis is an accurate technique utilized for the measurement of the velocity and motility parameters of mithun semen. In a span short time period high numbers of spermatozoa/semen can be estimated individually (Verstegen et al., 2002).

As compared with the report of early authors, the velocity and motility parameters were highly varied in mithun bulls (Farrell et al., 1996). Velocity and motility parameters had shown distinct differences with the parameters like time of collection, energy stores of sperm, age, time between ejaculations, viscosity, pH, ionic concentration, presence of agglutins and detergents, osmolarity, temperature in the seminal plasma and also the availability of the mineral elements like copper, zinc, manganese, and other hormones, PGs, etc. (Blasco, 1984).

The sperm mobility character can be contributed to specific sperm velocity parameters of individual sperm as determined by computer assisted sperm analysis. The velocity and motion parameters like VSL, LIN and BCF are very important for the overall characters of spermatozoa in bulls and as these parameters were significantly associated with sperm mobility and velocity. Experiment carried on for various types of sperm mobility representing VCL, VSL, VAP, LIN, STR, WOB, ALH, BCF and others have been shown in Table 1. The parameters VSL, VAP, VCL, LIN, and BCF were also significantly higher for the ejaculates collected from the normal intact mithun bulls as high mobility as compared with the cryptorchid mithun males. Thus, high-mobility sperm swim faster and straighter than did low-mobility sperm. This is due biologically significant as because the sperm mobility and velocity phenotype on the basis of investigation with the ejaculates is diagnosis of fertility (Froman & Feltmann, 1998; Froman et al., 1999).

The result of the present study revealed that the percent of total motility and forward progressive motility were significantly higher in intact mithun bulls than cryptorchid mithun bulls. Similarly, velocity parameters were significantly higher in intact mithun bulls than cryptorchid mithun bulls. Similar report was not available in cattle and other species to compare the present study results.

Table 2 Correlation between the motility and velocity parameters of sperm of normal intact mithun semen.

	FPM	TM	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
FPM	1.00	0.74*	0.67*	0.69*	0.72*	0.55	0.70*	0.65*	0.65*	0.66*
TM		1.00	0.95*	0.93*	0.94*	0.73*	0.82*	0.72*	0.93*	0.95*
VCL			1.00	0.95*	0.97*	-0.85*	-0.85	0.65*	0.95*	0.94*
VSL				1.00	0.95*	-0.76*	-0.80*	0.66*	0.94*	0.93*
VAP					1.00	-0.84*	-0.88*	0.78*	0.97*	0.94*
LIN						1.00	0.77*	-0.45	-0.76*	-0.75*
STR							1.00	-0.93*	-0.85*	-0.86*
WOB								1.00	0.74*	0.76*
ALH									1.00	0.93*
BCF										1.00

*Correlation coefficient were significant, p< 0.05

However, the motility and velocity parameters were measured by CASA in domestic animal species such as crossbred cattle (Perumal, 2008; Perumal et al., 2011), Bali cattle (Sarsaifi et al., 2013), buffalo (Anil Kumar et al., 2011; Koonjaenak et al., 2007), sheep (Kumar et al., 2010), goat (Kozdrowski et al., 2007), boar (Broekhuijse et al., 2012) and dog (Domosławska et al., 2013).

The cryptorchid mithun showed the decreased sperm motility & velocity and all motile spermatozoa were also non-progressive in nature as reported in other affected domestic livestock animals (Pinart et al., 1999). Most common reason of reduced motility and velocity in cryptorchid bull are abnormal spermatogenesis and spermiogenesis, which alter the ultrastructure of sperm tail (Gopalkrishnan et al., 1995), affects the secretory activity of accessory sex glands and epididymal epithelium, which changes the biochemical structure of epididymal fluid, leads to poor development of sperm motility and velocity in the epididymal duct (Yeung et al., 1993) and vitiated (impaired) functionality of the accessory sex glands may not only produces changes in the ejaculate volume, but even changes in the semen osmolality that are responsible for alterations of motility and velocity of spermatozoa (Gopalkrishnan et al., 1995).

Semen samples with high forward progressive motility and total motility had significantly higher positive correlation with velocity parameters in normal intact mithun bull (Table 2). The ejaculates with high PFM and TM had significantly higher average path velocity, progressive velocity and track speed. This was similar to the findings of earlier reports (Anil Kumar et al., 2011; Perumal et al., 2011; Perumal, 2008) for path velocity. The VAP was highly significant and correlated positively with track speed, progressive velocity and ALH. Similarly, the highly significant positive correlation was observed between VAP, VCL, VSL and ALH, between VCL and VSL and between ALH with VSL, VAP and VCL suggested that the velocity ad motility parameters were correlated, interrelated and associated among the velocity parameters and with amplitude of lateral displacement. Beat cross frequency was significantly and positively highly correlated with ALH. Anil Kumar et al. (2011), Perumal et al. (2011) observed that a positive correlation was similar to the present experiment between beat cross frequency and ALH. BCF and ALH indicating the head behaviour of the sperm are significantly variable and average value of these parameters were observed within the range from the previous studies. The authors also found that there was a highly significant negative correlation observed between STR and WOB and a similar result was observed in the present study. In cryptorchid mithun semen, there was positive correlation between the VCL and, VSL, VAP, ALH, BCF. Similarly, in normal intact bull, VCL is positively correlated with VSL, VAP, STR, ALH and BCF and negatively correlated with WOBB (Table 3) (Anil Kumar et al., 2011; Perumal et al., 2011).

Table 3 Correlation between the motility and velocity parameters of sperm of unilateral cryptorchid mithun semen.

	FPM	TM	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
FPM	1.00	0.59	0.04	0.12	-0.08	-0.08	-0.12	-0.06	0.11	-0.15
TM		1.00	0.26	0.15	0.16	-0.32	-0.22	-0.38	0.13	0.22
VCL			1.00	0.86*	0.92*	-0.24	0.04	-0.52	0.93*	0.85*
VSL				1.00	0.89*	0.27	0.11	-0.58	0.86*	0.64
VAP					1.00	-0.12	0.13	-0.33	0.97*	0.81*
LIN						1.00	0.85*	0.62	-0.12	-0.43
STR							1.00	0.17	0.22	-0.19
WOB								1.00	-0.43	0.94*
ALH									1.00	0.81*
BCF										1.00

*Correlation coefficient were significant, p< 0.05

Spermatozoa with progressive forward motility along with certain velocity parameters are essential for the spermatozoa to reach the site of fertilization and achieve fertilization. Parameters such as progressive forward motility, VSL, VCL, ALH and LIN were associated with the fertility of the bull (Farrell et al., 1996; Perumal et al., 2011). The VSL is the determination of average path velocity of spermatozoa head along a straight line from its starting position to last position measured. The VCL is the average path velocity of the spermatozoa head along its actual trajectory. The percentage of LIN is the ratio between VSL and VCL. The ALH is the average value of the extreme side-to-side movement of the spermatozoa head in each beat cycle. A very high ALH and VCL in the spermatozoa suggested the higher bending of the mid piece and having a higher ALH. This gives importance the hyperactivation of the spermatozoa.

The high energy state of the germ cells is the hyperactivation, which is essential for sperm penetrating through the cervical mucus and thus fusing with the oocytes (Aitken et al., 1985). Spermatozoa velocity and motility parameters indicate indirectly their mitochondrial membrane potential. In cattle species, some specific motion and velocity parameters have been reported to be correlated to fertility (Budworth et al., 1988; Farrell et al., 1996). However, the threshold levels for velocity and mobility parameters have not yet been standardized to understand a general consensus purpose . Furthermore, the CASA parameter, linearity or linear motility is significantly higher suggests that the sperm cells has higher fertilization potential rate as compared to the total as well as forward progressive motility (Cremades et al., 2005) and ejaculates contains such sperm has higher fertility percentage and pregnancy rates after artificial breeding (Farrell et al.,1998).

Recent findings suggested that determination of motile spermatozoa in a ejaculate may not be recognized as a reliable and useful index in semen assessment and conservation. The objective and subjective estimation of other sperm velocity and motion parameters obtained from observations of individual sperm cells assessed by CASA have been seen to be more efficient in diagnosing the fertility potential of ejaculates (Mortimer, 1994). All these CASA parameters are probably essential for the progression and forward movement of sperm into estrus cervical mucus and the passage and/or penetration of *zona pellucida* and successful fertilization (Verstegen et al., 2002). Fertilization rates of oocytes in *in vitro* have been showed to associated positively with sperm velocity and motility (Donnely et al., 1998).

In cattle species, sperm velocity parameter is highly correlated with the 59 d non-return rate (Farrell et al., 1998). VCL and BCF were significantly higher indicates the spermatozoa have higher percentage that penetrated in penetration assay than the sperms failed to penetrate successfully in experiment related with human beings (Fetterlof & Rogers, 1990). The computerized sophisticated technique is not only prognoses the semen fertility and but also can be used as a tool to study the effects of different *in-vitro* protocols on sperm velocity and motility as well as the sperm hyperactivation phenomenon (Farrell et al., 1993) and similar observation was found in the current study.

Based on the current study, it was concluded that unilateral cryptorchidism causes subfertility/infertility in males due to significant alterations in the seminal and scrotal & testicular parameters of mithun. Results revealed from the study that most of the important sperm velocity and motility parameters of computer assisted sperm analyser were significantly lower in ejaculates of cryptorchid mithun bulls in comparison to the ejaculates of normal intact mithun bulls. Due to the genetic hereditary nature, cryptorchid or monorchid diagnosed bulls never be used for breeding purposes either natural or AI program.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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