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Sensory Attributes of Namakkal Quail-1 Meat

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Abstract To improve the meat quality of Japanese quail (*Coturnix coturnix japonica*), a new strain was developed by Tamilnadu Veterinary and Animal Sciences University named “Namakkal quail-1”. The purpose of the study is to know the sensory quality of the Namakkal quail-1. 24 Birds including 12 males and 12 females of 4th and 6th week birds were procured from Poultry farm Complex of Department of Poultry Science, Veterinary College and Research Institute, Namakkal and slaughtered as per the standard slaughter procedures after 4 hours of resting interval at the Department of Livestock Products Technology (Meat Science), Veterinary College and Research Institute, Namakkal. The sensory properties like colour, flavour, juiciness, tenderness and overall acceptability were estimated. Analysis of variance showed that age and sexes of the birds no significant effect on colour, flavour, juiciness, tenderness and overall acceptability of meat. But Namakkal quail-1 meat score higher than Japanese quail, Chicken, Duck and Geese meat in 9 point hedonic scale. It is concluded that Namakkal quail-1 meat is tastier than other poultry meat.
Keywords *Colour; Flavour; Namakkal Quail-1; Overall Acceptability; Tenderness*

1. Introduction

Quail meat is a delicious white meat with extremely low skin fat and cholesterol value. It is rich in micronutrients and a wide range of vitamins including B Complex, Vitamin E and K (Imchel, 2013). The percentage content of edible meat in Japanese quail is very high. Breast, leg and wing contain 37.3-38.7 per cent, 22.7-24.4 per cent and 35.9-37.8 per cent body weight, respectively. The protein, moisture and fat contents of raw quail meat are 20.54 per cent, 73.93 per cent and 3.85 per cent respectively (Panda et al., 1987). Quail meat is tastier than chicken and has less fat content. It promotes body and brain development in children (Imchel, 2013). It is also a good source of phosphorus, iron and copper. Many newer strains have been developed recently. Tamil Nadu Veterinary and Animal Sciences University developed a new quail strain named as “Namakkal Quail-1” during the year 2006. It is a meat type commercial hybrid and is produced by four way crossing of Japanese quail.

2. Materials and Methods

24 Birds including 12 males and 12 females of 4th and 6th week birds were procured from Poultry farm Complex of Department of Poultry Science, Veterinary College and Research Institute, Namakkal and starved for 4 hours and slaughtered as per the standard procedure in department of Meat Science and Technology, Veterinary College and Research Institute, Namakkal. Quails were slaughtered by decapitation. Following a 5 min bleeding time, feathers were removed along with skin by hand. Carcasses were eviscerated and deboned manually. The sensory properties of cooked breast meat samples were assessed by subjecting to a sensory analysis for colour, flavour, juiciness, tenderness and overall acceptability by semi trained sensory panel consisting of five members. The meat samples for sensory evaluation were coded and pressure cooked at 10 psi pressure for 10 minutes. The cooked samples were cut into small cubes of approximately 1.5 cm and served to the panellist with a nine point hedonic scale (Cover et al., 1962) as given in the score sheet. Then the results were analyzed statistically.

Table 1: *Mean (± S.E.) Sensory attributes of 4 and 6 weeks old Namakkal Quail-1*

Parameters	4th week			6th week		
	Male	Female	Overall Mean	Male	Female	Overall Mean
Colour	7.58±0.09	7.63±0.09	7.60±0.06	7.66±0.11	7.68±0.09	7.67±0.07
Flavour	7.65±0.13	7.43±0.17	7.54±0.11	7.58±0.08	7.63±0.18	7.60±0.09
Juiciness	7.78±0.11	7.73±0.13	7.75±0.08	7.43±0.13	7.53±0.20	7.48±0.11
Tenderness	7.61±0.14	7.53±0.12	7.57±0.09	7.68±0.27	7.48±0.13	7.58±0.14
Overall acceptability	7.65±0.10	7.73±0.08	7.69±0.06	7.66±0.10	7.78±0.10	7.72±0.07

No significant difference found

3. Results and Discussion

3.1. Colour

Colour is the first criterion consumer's use to judge meat quality and acceptability. Colour is mainly influenced by myoglobin content and nature, the composition and physical state of muscle and meat structure (Renerre, 1986). The sensory panel rating for colour ranged from 7.58 to 7.68 for 4th and 6th week birds. According to Akinwumi et al. (2013), in 9 point hedonic scale the colour scores were 7.2 for geese, 6.6 for chicken and 6 for Japanese quail, respectively (Table 1). The geese scored higher score in terms of colour followed by chicken and then Japanese quail. On comparing this to Namakkal quail-1, it scores higher. In the present study, colour of the meat had not significantly ($P>0.05$) affected by age of the birds.

3.2. Flavor

Flavour of the meat mostly depends on sex and age of the birds. As the age increases, the flavour of the meat also increases due to the increase in fat content (Lawrie, 1998). In the present study, flavour of the meat had not significantly ($P>0.05$) affected by age and sex of the birds. The score for flavours ranges from 7.54 to 7.60 for 4th and 6th week Namakkal quail-1 meat (Table 1). On comparison it was 6.0 for chicken, 6.9 for quail, 5.0 for geese and 5.9 for duck (Akinwumi et al., 2013). The Namakkal quail scores higher for flavour followed by Japanese quail, chicken, duck and geese. On contrary to this present study, in duck meat, the flavour of female meat scores higher than the male birds (Omojola, 2007).

3.3. Juiciness

Juiciness of meat is directly related to the intramuscular lipid and moisture content of the meat. In combination with water, the melted lipids constitutes a broth which when retained in meat is released upon chewing. In the present study the sex and age had no significant (P>0.05) effect on the juiciness of the meat. On contrary to this, Omojola (2007) reported that sex had significant effect on juiciness of duck meat. The Female bird meat scores higher than the male bird meat. The juiciness of the meat mostly depends upon the water holding capacity and cooking loss of the meat. Since the Namakkal quail-1 meat had less fat and no change in water holding capacity of meat, there was no significant change in juiciness of meat. Namakkal quail-1 meat found to have highest juiciness with score of 7.7 to 7.48 (Table 1). When compared to Japanese quail meat (6.4), chicken meat (5.1) and geese meat (3.8) (Akinwumi et al., 2013).

3.4. Tenderness

Tenderness is regarded as the most important sensory attribute affecting the meat acceptability. In present study, tenderness was not significantly (P>0.05) affected by age and sex of the birds. On contrary to this in duck, Omojola (2007) reported that tenderness was affected by sex of the birds. The male duck meat scores higher for tenderness than females. The age of the birds also had significant effect on tenderness of meat. The meat from 33 days old bird score higher than the 42 days old Japanese quail birds (Wilkanowska and Kokoszyński, 2011). According to Akinwumi et al. (2013), Japanese quails found to have the highest tenderness score of 6.10, followed by chicken (5.4), but the geese had significantly lower score. On comparison to all the above, Namakkal quail-1 meat scored high for tenderness.

3.5. Overall Acceptability

The overall acceptability of the meat determines the quality of meat. In the present study the sex and age of the birds had no significant (P>0.05) effect on overall acceptability of Namakkal quail-1 meat (Table 1). This was agreed with Omojola (2007) reported that overall acceptability were not affected by sex of the bird in duck meat.

4. Conclusion

Even though the sensory characteristics showed no significant difference between age and sex of the birds, the Namakkal quail-1 meat scored higher when compared to duck, geese, Japanese quail and chicken meats.

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Research Article **Open Access**

Complete Genome Characteristics of Porcine circovirus Type 2 (PCV2) Isolates from Papuan Pigs, Indonesia

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Abstract Porcine circovirus type 2 (PCV2) has been recognised as an important pathogen in the pig industry world-wide. The virus was recently identified in Papuan pigs, yet information about the characteristics of Papuan PCV2 isolates is limited. The aim of the present study was to characterise the complete genome of Papuan PCV2 isolates. Viral DNA was isolated from 13 blood samples of village pigs from the Jayawijaya region. Four of the PCR positive samples were selected for full genome sequencing. Neighbour Joining phylogeny with P-distance model showed that the four Papuan PCV2 isolates belong to genotype PCV2-IM3. Sequence identity analysis of the Papuan PCV2 genomes further showed 99.5% to 99.6% similarity with a Chinese PCV2-IM3 reference strain. The current study revealed that genotyping based on the ORF2 sequence resulted in a substantially different characterisation of PCV2 genotypes compared to classification based on the complete genome sequences. Furthermore, the study showed that the topology of the PCV2 phylogeny based on the ORF2 sequence was different from the topology based on capsid proteins. Genotyping using the complete genome, ORF2 region or capsid protein sequences resulted in substantial variance in the classification of the PCV2 isolates. The clinical consequence of these different genotyping methods needs further study.
Keywords *Papua; Pigs; ORF2; Neighbour Joining; PCV2-IM3 Genotype*

1. Introduction

Porcine circovirus type 2 is an emerging pathogen causing economic loss in the pig industries worldwide. Infection with PCV2 manifests in various clinical outcomes, termed porcine circovirus associated diseases (PCVD) and characterized by a respiratory disease complex, dermatitis and nephropathy syndrome or post weaning multisystemic wasting syndrome (PMWS). In order for PCVD to be produced, co-infection with PCV2 and other factors is required (Baekbo et al., 2012). Reported PCVD co-factors included porcine reproductive and respiratory syndrome (PRRS), porcine parvovirus (PPV), Swine Hepatitis E virus (HEV), *Mycoplasma hyopneumoniae, Salmonella spp.* Or *Metastrongylus elongatus* (Allan et al., 2000; Ha et al., 2005; Kennedy et al., 2000; Marruchella et al.,

2012; Opriessnig T. et al., 2004; Yang et al., 2015) as well as environmental stressors, such as changes in temperature and high stocking density (Patterson et al., 2015).

Genotype variations of PCV2 virus have been observed; one widely recognised method for the classification of PCV2 genotypes is the Neighbour Joining (NJ) phylogenetic tree approach with pairwise proportional difference of nucleotides (P-distance) model, using the ORF2 gene region or the complete genome as target sequences. The cut off value of proportional nucleotide diversity for genotype demarcation is 0.035 in ORF2 based analysis or 0.02 when the complete genome is used (Segales et al., 2008).

Using the NJ method with P-distance model, four major genotypes of PCV2 have been established, namely PCV2a, PCV2b, PCV2c and PCV2d. Further, a few intermediate (IM) groups; IM1-IM4 have also been reported (Xiao et al., 2015). The most prevalent circulating genotype in farmed pigs has been PCV2b, followed by PCV2a. Genotype PCV2d may be a new emerging genotype in farmed pigs and recently has been identified amongst herds with vaccination failure in USA, Korea and Brazil (Segales, 2015; Xiao et al., 2015). Genotype PCV2c consists so far of only four strains (Franzo et al., 2015). Intermediate clades have consisted of less than fifty strains (Xiao et al., 2015).

Commercial vaccines against PCV2 have been developed based on the capsid protein of ORF2 genes of the PCV2a genotype and known to be protective against infections with PCV2a and PCV2b (Segales, 2015). However, the observation that PCV2d can be retrieved from vaccinated pigs invites a discussion on the cross protection provided by commercial vaccines for PCV2d (Segales, 2015; Xiao et al., 2015). Furthermore, the level of cross protection of available vaccines against PCV2c and intermediate groups is unknown.

To date, only two papers have been published on the topic of PCV2 from Indonesia, one from Western Indonesia (Manokaran et al., 2008) and the other one was from Papua (Nugroho et al., 2015). Papua province has the fifth highest pig population in Indonesia (Siagiaan, 2014) and village pigs are important livelihood assets for the Papuan community (Nugroho et al., 2015). The aim of the present study was to characterise the Papuan PCV2 isolates based on either the complete genome sequences, ORF2 region and capsid protein using NJ method. This information will contribute to a better knowledge of the epidemiology of PCV2 infections in Papua, Indonesia.

2. Materials and Methods

2.1. PCR and Complete Genomes Assembly

Total DNA from thirteen serologically PCV2 positive blood samples was used in present study. The samples were retrieved from village pigs from Jayawijaya Region, Papua, Indonesia. The two pairs of primers used in the current full genome sequencing study were 20 base pair (bp) length respectively, reported previously (Fenaux et al., 2000). The first pair of primers, CV1 and CV2, amplified a 989bp fragment. The CV1 primer is 5'-AGGGCTGTGGCCTTTGTTAC-3', situated at position 1336-1355 in PCV2 genome and CV2 is 5'-TCTTCCAATCACGCTTCTGC-3' located at position 536-556 of PCV2 genome. Second set of primers, CV3 and CV4, amplified a 1,092-bp fragment. The sequence of CV3 and CV4 were 5'-TGGTGACCGTTGCAGAGCAG-3' and 5'-TGGGCGGTGGACATGATGAG-3' respectively, positioned at 452-471 and 1525-1544 in PCV2 genome. The PCR products of PCV2 DNA resulting from amplification using these two pairs of primers overlap at positions 452-536 and 1355-1544 in PCV2 genome.

Amplification of PCV2 genomes was conducted using conventional PCR. Each of the PCR mix contained 2.5 µL of 10X buffer, 0.5 µL of 10 mM dNTP, 1 µL of 50 mM $MgSO_4$, 1 µL of each of primers, 0.2 µL Platinum *Taq*DNA Polymerase High Fidelity (Invitrogen, USA) and 3 µL DNA. The

PCR cycle was done using Biorad T-100 thermocycler (Bio-Rad, USA) and programs for both primer pairs consisted of an initial denaturation of 94^{o}C for 2 min, followed by 35 cycles of consecutive denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec and extension at 68^{o}C for 1 min. The PCR was completed at a final extension of 68^{o}C for 5 min. Only PCR products that showed clear target bands with minimal additional lower bands were selected for further sequencing.

The selected sets of PCR products were sequenced using the Sanger method at the Australian Genome Research Facility (AGRF, Adelaide, Australia). The sequences were assembled based on the overlapping sequences for each sample compared to a few selected sequences available at NCBI (http://www.ncbi.nlm.nih.gov/).

2.2 Genotyping and Capsid Proteins Analyses

In order to infer the genotype of the Papuan PCV2 isolates, the selected Papuan complete genomes, as well as their ORF2 genes were aligned with 1390 PCV2 sequences obtained from GenBank, using ClustalX 2.1 (Larkin et al., 2007). Phylogenetic trees were subsequently constructed based on the Neighbour Joining method and P-distance model with 1000 bootstraps pseudo replication, in *MEGA* 5 software (Tamura et al., 2011). The phylogenetic tree was generated using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and genotyping using the full genome sequence was compared with genotyping using ORF2 sequence. For the analysis of the capsid protein, amino acids coded by the ORF2 genes of the Papuan isolates and 1390 PCV2 sequences obtained from GenBank were translated into Bioedit (Hall, 1999) and aligned in ClustalX 2.1 (Larkin et al., 2007). NJ P-distance phylogenetic trees were then constructed to compare the groups of capsid proteins amongst PCV2 strains.

Furthermore, the P-distance matrix of the Papuan isolates and selected reference strains of different genotypes were calculated in MEGA 5 (Tamura et al., 2011). P-distance analyses were performed at the level of the complete genome, ORF2 genes as well as at the predicted amino acid sequences of the capsid proteins. Additionally, a sequence identity matrix (SIM) and classical dendograms of complete genomes was constructed for the Papuan PCV2 isolates and a few selected reference strains of PCV2-IM3, PCV2a, PCV2b, PCV2c and PCV2d genotypes.

3. Results

3.1. Complete Genomes Description and Genotyping

Four out of positive 13 samples that showed clear PCR products on agarose gel were selected for complete genome sequencing. The full genome of the four PCV2 isolates comprised of 1,767 bp length. The nucleotide divergence of the Papuan isolates ranged from 0.002-0.004. The size of the ORF1 sequence was 945 bp, situated at nucleotide position 51-995, encoding a protein of 314 amino acids (aa) size. The size of the ORF2 sequence was 705 bp, at position 1734-1030, encoding a protein of a size of 234 aa.

The complete genomic phylogenetic tree suggested that the Papuan PCV2 isolates are grouped together with strains belong to the PCV2-IM3 genotype. The members of this genotype, apart from the Papuan isolates are Brazilian isolates (KJ094602, KJ094605, KJ094606), the Croatian isolate (HQ591381), the Indian isolate (LC004753) and a Chinese isolate (HM776452) (Figure 1a), with a mean pairwise genetic distance of 0.018 (SE: 0.002, range: 0.001-0.035). Genotyping based on the ORF2 gene however, excluded the Brazilian strains (KJ094602, KJ094605, KJ094606) from the genotype, but included a further 39 Chinese isolates and retained the Croatian (HQ591381), Indian (LC004753) and Chinese (HM776452) isolates in the IM3 genotype. This comparison resulted in a larger sized group that comprised 46 strains (Figure 1b) with a genetic distance of 0.033 (SE: 0.003,

range: 0.001-0.088). The P-distance of the Papuan strains and IM3 genotypes, based on the complete genome and ORF2 genes, was 0.004 and 0.007 respectively, lower than the threshold of 0.02 and 0.035, respectively supporting the position of Papuan PCV2 isolates in the IM3 genotype (Table 1). SIM (Table 2) and classical dendogram (Figure 2) further confirmed that the Papuan isolates belong to the PCV2-IM3 genotype. Additionally, variation within the Papuan isolates occurs in the intergenic region at position 42, in the ORF1 gene at positions 131, 389, 405, 604, 910, and in the ORF2 gene zat positions 1558, 1561, 1591 and 1619.

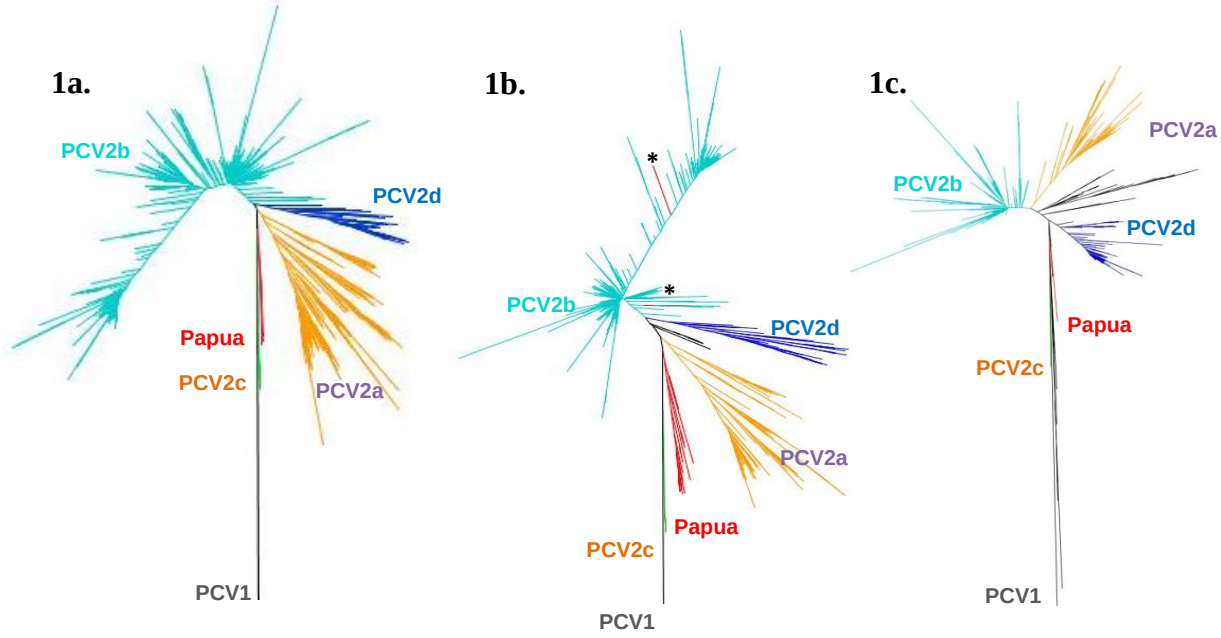

Figure 1: *Evolutionary relationships of the Papuan PCV2 isolates and other genotype strains. 1a. Phylogenetic tree based on complete genome (1682 nt), 1b. Phylogenetic tree based on ORF2 gene (664 nt) and 1c. Phylogenetic tree based on Capsid protein (222 aa). The analysis involved 1,395 nucleotide sequences. PCV1 was included as an outgroup. Evolutionary analyses were conducted in MEGA5. Color explanation: Orange, PCV2a; Light blue, PCV2b; light green, PCV2c; deep blue, PCV2d; red, Papuan and IM3 genotypes; black, PCV1.* (asterix) shows the position of Brazilian strains KJ094602, KJ094605 and KJ094606 which move from the Papuan group in Figure 1a. to PCV2 b genotype group in Figure 1b.*

Table 1: *Genetic divergence of Papuan isolates and different PCV2 genotypes, calculated using Pairwise distance method with 1,000 bootstrap pseudo replication. Analyses involved complete genomes, ORF1 genes, ORF2 genes and amino acid of capsid protein sequences. Papuan isolates show lowest genetic divergence from the PCV2-IM3 reference strains compared to its genetic divergence with other genotypes*

Region of DNA sequence	Within Papuan isolates (SE) n=4	Between Papuan isolates and other genotypes				
		PCV2-IM3	PCV2a	PCV2b	PCV2c	PCV2d
		HM776452	AF055392	AF055394	EF524532	AY181946
Complete genome (1735 nt)	0.003 (0.001)	0.004	0.04	0.036	0.047	0.041
ORF1 gene (933 nt)	0.004 (0.001)	0.002	0.014	0.022	0.02	0.028
ORF2 gene (691 nt)	0.002 (0.001)	0.007	0.081	0.058	0.086	0.063
Capsid protein (231 aa)	0.002 (0.002)	0.01	0.083	0.057	0.096	0.049

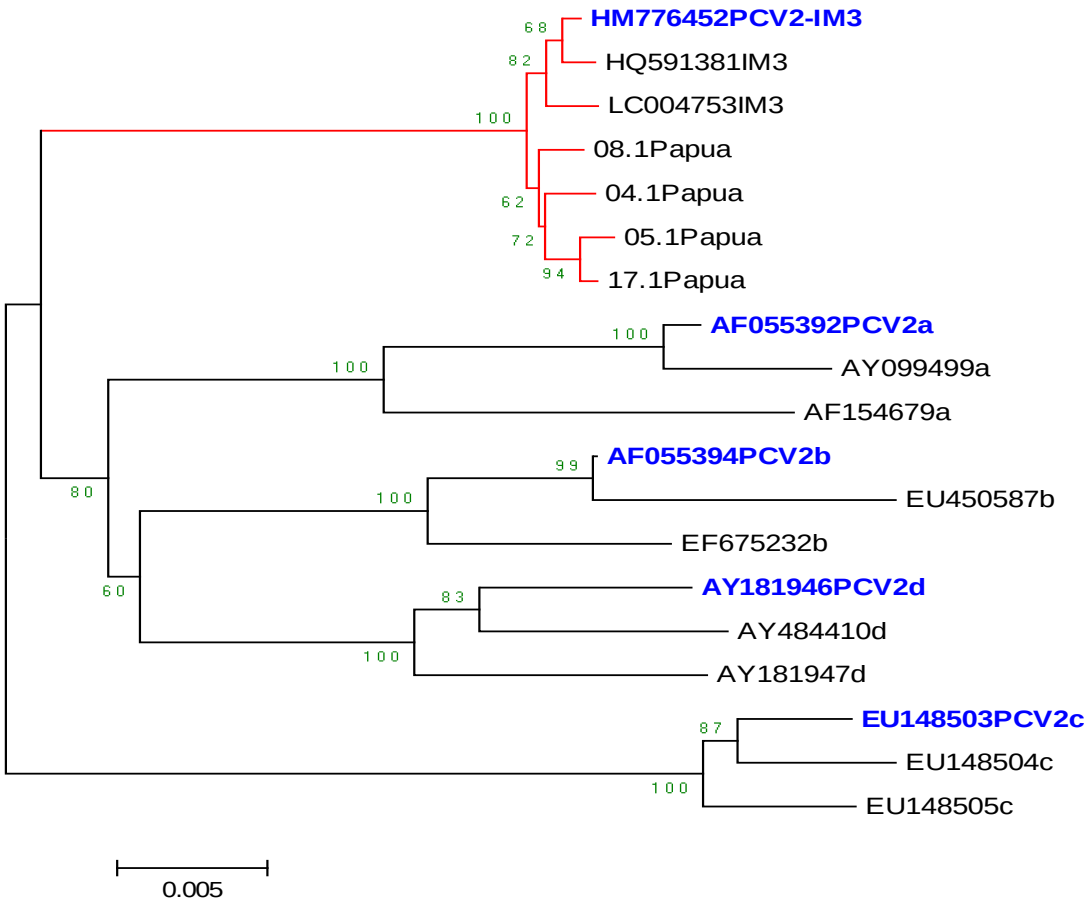

Figure 2: *Dendogram of Papuan PCV2 isolates and selected reference strains from different genotypes. The analysis involved 19 nucleotide sequences. There were a total of 1765 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Papuan PCV2 isolates are grouped in the clade of PCV2-IM3 genotype*

Table 2: *Sequence identity matrix (SIM) of complete genomes of Papuan PCV2 isolates and selected reference strains from different genotypes. Papuan isolates show high similarity of 99.5-99.6% with PCV2-IM3 genotype reference strain*

PCV2 strains	04.1Papua	05.1Papua	08.1Papua	17.1Papua	PCV2IM3	PCV2a	PCV2b	PCV2c	PCV2d
					HM776452	AF055392	AF055394	EU148503	AY181946
04.1Papua									
05.1Papua	99.6%								
08.1Papua	99.6%	99.6%							
17.1Papua	99.6%	99.8%	99.6%						
HM776452PCV2-IM3	99.6%	99.5%	99.6%	99.6%					
AF055392PCV2a	95.7%	95.7%	95.7%	95.7%	95.9%				
AF055394PCV2b	96.2%	96.2%	96.4%	96.2%	96.3%	96.2%			
EU148503PCV2c	95.0%	94.9%	95.1%	95.0%	95.0%	94.6%	95.3%		
AY181946PCV2d	95.8%	95.8%	95.8%	95.8%	95.9%	95.8%	96.8%	95.1%	
AY193712PCV1	76.6%	76.6%	76.6%	76.5%	76.4%	76.1%	76.5%	76.2%	75.7%

3.2. Predicted Capsid Protein Analyses

The topology of PCV2 phylogeny based on capsid proteins was slightly different from the topology of the PCV2 phylogeny based on the ORF2 gene. In the capsid proteins based phylogeny, PCV2b was located closer to PCV2a, while when analysis was based on the ORF2 gene, PCV2b is the sister clade of PCV2d. The capsid proteins of the Papuan isolates and PCV2-IM3, however remain in consistent topology as immediate descendants of PCV2c (Figure 1c). The amino acid divergence of the capsid protein of Papuan PCV2 isolates was lowest with PCV2d compared to their amino acid divergence with other major PCV2 genotypes. The amino acid sequences of the capsid proteins of the Papuan isolates differed only at position 39 (Arg39Lys).

3.3. GenBank Accession Number

The complete genomes of the Papuan PCV2 isolates used in this study can be retrieved from GenBank with the accession numbers KT369067, KT369068, KT369069 and KT369070.

4. Discussion

PCV2 is an important disease in the pig industry world-wide, causing significant economic loss (López-Soria et al., 2014). In Eastern Indonesia, where traditional pig husbandry practices are predominant, study of the infection with this pathogen is rare. In our current study, we characterized the complete genome of four Papuan PCV2 isolates obtained from village pigs. The Papuan PCV2 isolates in the current study can be grouped with the PCV2-IM3 genotype using NJ phylogeny with P-distance model.

Strains belonging to the PCV2-IM3 genotype have been isolated from pigs across Brazil, China, Croatia and India. In a Brazilian study (Franzo et al., 2015) and our current investigation strains were retrieved from feral pigs. There is no information available as to the host characteristics of the isolates from China and Croatia. PCV2-IM3 genotype might be actually more prevalent in feral pigs rather than in modernly farmed pigs.

The current study demonstrated that there were substantial differences between the results of genotyping based on the ORF2 region and the classification using complete genome sequences. Analysis using the complete genome showed that PCV2-IM3 has lower genetic diversity compared with genotyping using the ORF2 gene. Furthermore, in the current analysis, three Brazilian reference isolates, which belong to the PCV2-IM3 genotype in the complete genome, based analysis grouped with PCV2b when the ORF2 gene was used for genotyping. ORF2 was perceived as the region's representative of the complete genome variation and it has been suggested by others to use either the ORF2 region or full genome sequences in PCV2 genotyping (Segales et al., 2008). We suggest that complete genome sequences should always be used for genotyping of PCV2, as the methods to obtain complete genome of this small virus have largely been available.

A previous study reported that the three Brazilian reference isolates belong to the PCV2d genotype when the analysis involved only a small number of samples consisting of just 36 complete genome sequences (Franzo et al., 2015). Similarly, a study indicated that a phylogenetic analysis using just 48 strains resulted in a topology different from an analysis using a large (n=1, 680) set of PCV2 reference strains, noting the importance of large sample size when determining the classification of PCV2 (Xiao et al., 2015). Not only a different number of samples, but also a different set of sequences within the same number of samples used in the construction of phylogeny tree may produce a different topology. A guideline to genotyping PCV2 using a smaller number of samples may require further study.

Monovalent vaccines based on the capsid protein of either PCV2a or PCV2b genotype have been efficacious to prevent infections with either PCV2a, PCV2b or their mixed infections (Jeong et al., 2015; Opriessnig et al., 2013; Segales 2015). The PCV2d strains on other hand, were isolated from herds that had been vaccinated with PCV2a vaccines (Segales, 2015). Our current study showed that ORF2 of PCV2d was more similar to PCV2b than PCV2a, while ORF2 of PCV2a was highly similar to PCV2b compared to other genotypes. This might explain partly the cross reaction of PCV2a and PCV2b vaccines, and the failure of PCV2a vaccine to protect infection with PCV2d genotype. In the case of Papua PCV2 isolates, the similarity of their ORF2 genes with PCV2b is comparable to with PCV2a. However, the capsid protein of Papuan PCV2 isolates was more similar to PCV2d. The implication of these phenomena on the efficacy of commercial vaccines against Papuan PCV2 isolates requires further study.

5. Conclusion

In conclusion, the present study showed that Papuan PCV2 isolates belong to the PCV2-IM3 genotype. Distribution of this genotype currently encompasses China, Croatia, India and Indonesia. Genotyping using complete genome, ORF2 region or capsid protein sequences resulted in substantial difference in PCV2 strains classification. The clinical implication of these different genotyping methods requires further investigation.

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Conflicts of Interest

All authors declare no conflict of interest.

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Research Article **Open Access**

Identification of Heparin Binding Proteins in Frozen-thawed Capacitated/Acrosome-reacted Spermatozoa and Their Relationship with Fertility of Buffalo Bull Semen

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Abstract The present was conducted to separate heparin binding proteins (HBP) of capacitated sperm extracts and determine their relationship with acrosome reaction and semen fertility of bulls (n=30). Fourteen immunoreactive bands in capacitated spermatozoa of bulls (135, 100, 75, 70, 65, 55, 48, 45, 33, 31, 26, 24, 20 and 18 kDa) were detected in western blots following incubation with anti-HBP antibody. The frozen-thawed semen was evaluated for first service conception rate (FSCR) and percent acrosome reaction and linked to HBP. In capacitated spermatozoa, bulls positive for 70, 31, 20 and 18 kDa had significantly higher ($P < 0.05$) FSCR (42.3 ± 5.1 vs 32.9 ± 3.9%, 45.0 ± 6.5 vs 34.1 ± 3.5%, 60.0 ± 10.0 vs 35.2 ± 3.3% and 42.9 ± 5.1 vs 31.9 ± 3.7%), respectively, as compared to their negative counterparts. Although non-significant, FSCR was reasonably higher ($P > 0.05$) in bulls positive for 135 and 100 kDa HBP than in their negative contemporary mates and exhibited an increase of 7.4% for the two proteins. A significantly higher ($P < 0.05$) rate of acrosome reaction was recorded in bulls with HBP of 135, 100, 70 and 18 kDa in comparison to their contemporary mates with a difference of 7.4, 7.4, 8.7 and 9.4%, respectively. Alternatively, FSCR and acrosome reaction of bulls with 75, 55 and 48 kDa were lower ($P < 0.05$) than their corresponding counterparts. In conclusion, HBP of 135, 100, 70 and 18 kDa did seem to activate *in vitro* sperm acrosome reaction and subsequent fertility of bulls.

Keywords *Acrosome Reaction; Buffalo Bull; Capacitation; HBP; FSCR; Semen*

1. Introduction

The heparin binding proteins (HBP) are produced by male accessory sex glands and upon ejaculation bind to the sperm [1]. The HBP bind to sperm membrane choline phospholipids and capacitation factors viz. heparin and glycosaminoglycans at ejaculation resulting in acrosome reaction, sperm oocyte fusion and fertilization [2]. HBP have predominately been linked to bull fertility potential. Five proteins with molecular weight of 18, 31, 33, 48 and 55 kDa have been identified as members of HBP family and are referred to as fertility-associated antigens [3]. Another HBP (24 kDa) was found to have an amino acid sequence related to tissue inhibitor of metallo-proteinases-2 and played an

important role in buffalo bull fertility [4]. Further, two proteins (BSP-A1 and BSP-A2) exhibited their binding capacity to heparin and could be used as biochemical markers to predict fertility potential of bulls [5]. The HBP protect sperm from stress of freezing and thawing and maintain intracellular protein homeostasis [6]. Characterizing functionally important HBP is a first step toward better understanding the modulating effects of seminal fluid on fertility of buffalo bulls. In addition, fertilization potential of spermatozoa *in vitro* is determined by capacitation / acrosome reaction status which involve different signal transduction pathways [7]. Keeping in view of above facts and also taken into consideration the deficit knowledge of HBP in buffalo bulls, the present study was designed to characterize HBP in capacitated frozen-thawed spermatozoa and determine their relationship with fertility of buffalo bull semen.

2. Materials and Methods

2.1. Procurement of Semen

Frozen semen (30 straws per bull) from thirty breeding Murrah buffalo bulls were procured from two government semen processing and freezing laboratories in the month of September having ambient temperature 30.6^0C and relative humidity 92%. The straws (0.25 ml each) were frozen from same ejaculate on same date and earmarked for the present study.

2.2. *In Vitro* Capacitation Acrosome Reaction

Semen from ten straws per bull was taken in 15 ml graduated tube and washed twice with the basic TALP medium (2 ml; 92.9 mM NaCl, 4 mM KCl, 25.9 mM $NaHCO_3$, Na_2HPO_4, 10 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM HEPES) by centrifuging at 1000 rpm for 5 minutes [8]. The sperm suspension was then re-suspended in the energy rich medium (0.5 ml; 10 ml basic TALP medium, 0.25% glucose, 0.6% bovine serum albumin, 10 mg of streptomycin and 100 µl of 0.1% stock solution of heparin), transferred in eppendorf tubes and placed in the incubator (5% CO_2) at 37°C for 6 h. Capacitation status was assessed at every 2 h interval for 6 h. At the end of every 2 h, a 10 µl sperm suspension was removed from the aliquot, smear was prepared, stained using Giemsa stain and assessed for acrosome reaction. The evaluation of the acrosome reaction was carried out by counting 200 spermatozoa from each smear under bright field microscope (100x). The spermatozoa showing complete sequence of capacitation events viz. swelling, vesciculation and shedding at 6 h were considered as acrosome-reacted.

2.3. Semen Fertility

The number of females inseminated per bull semen was ten. Therefore, ten mini straws from each bull were used for the field fertility trial. All the buffaloes (n = 300) enrolled for fixed time insemination program (October to April) were healthy, multiparous (2^{nd} to 5^{th} parity), recently calved (60-80 days earlier), free from physical problems, vaginal discharge and maintained under standard feeding and management systems. Prior to the start of breeding program, clinical assessment of genitalia was done ultrasonographically using a B-mode linear array trans-rectal transducer with 5/7.5 MHz interchangeable frequency (EXAGO, ECM, France) to visualize a cyclic CL twice at 10 days apart and rule out the possibility of reproductive tract infections, if any. The buffaloes were synchronized using double ovsynch protocol ($PGF2_{\alpha}$-GnRH-$PGF2_{\alpha}$-GnRH on day -2, 0, 7 and 9, respectively) followed by fixed time inseminations at 16 and 40 h after last GnRH injection, respectively. The pregnancy diagnosis was done on day 45 post-insemination and confirmed on day 60 using ultrasonography. The first service conception rate (FSCR) was calculated according to the following formula:

$$\text{FSCR (\%)} = \frac{\text{Number of buffaloes conceived after first insemination}}{\text{Total number of first services}} \times 100$$

2.4. Extraction of Sperm Proteins

The frozen-thawed semen (20 straws from each bull) were centrifuged at 3000 rpm for 10 minutes to separate out and discard dilutor. The remnant sperm pellet obtained from frozen-thawed semen was washed twice with PBS (pH 7.4), suspended in 1.0 ml of 2% SDS in 62.5 mM Tris-HCl (pH 8.0) containing protease inhibitors (Cocktail, SERVA). The sperm suspensions were sonicated at 4^{o}C (20 watts, three times for 20 seconds each) and then centrifuged at 10000 rpm for 15 minutes. The pellet was discarded and sperm extracts (SE) were collected and stored in 0.5 ml fractions of cryovials at -20^{o}C till further use.

2.5. SDS-PAGE and Immunoblotting

Exactly 100 µg of protein was fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel and 4% stacking gel. After electrophoresis, enzyme linked immuno transfer blot was done as per the method of Towbin et al. [9]. The frozen-thawed sperm extracts were reacted with anti-HBP (anti AZU-1, Sino Biological) to correlate such sperm specific proteins of buffalo bull spermatozoa with semen function tests and bull fertility. The blot images were captured on Syngene gel doc using Gene Snap image acquisition software and were analyzed for molecular weight and quantity by using Gene Tools gel analysis software (Syngene).

2.6. Statistical Analysis

The statistical analysis was performed with Statistical Package for Social Sciences (SPSS, version 16.0) program. The proportionality data (acrosome reaction and FSCR) were transformed using the arcsine transformation [asin (sqrt (percent/100))] with adjustment to allow for zero values. The mean ± SE were calculated using arcsine transformed data in the software. Duncan's multiple range tests and one way analysis of variance (ANOVA) was used for comparing the level of significance among the group of bulls of different gradients (bulls positive and negative for HBP). The mean ± SE were calculated using arcsine transformed data in the software. The minimum significant interaction was considered at 5% level.

3. Results and Discussion

3.1. Identification of HBP in Frozen-Thawed Capacitated Sperm Extracts by Immunoblotting

Blot images of protein bands in capacitated frozen-thawed sperm extracts of all 30 bulls have been shown in Figure 1. Anti-HBP (anti-AZU-1) recognized fourteen proteins of 135, 100, 75, 70, 65, 55, 48, 45, 33, 31, 26, 24, 20 and 18 kDa in capacitated spermatozoa of 10, 10, 13, 13, 17, 4, 4, 23, 16, 8, 5, 26, 3 and 14 bulls, respectively (Table 1). To the best of our knowledge, this is the first study to characterize HBP in frozen-thawed capacitated spermatozoa of buffalo bulls. The electrophoretic profiles showed polymorphism among individual semen samples ranging from 2-10 proteins in post-thaw capacitated semen. However, no individual tested bull had all bands in the capacitated sperm extracts. Therefore, qualitative differences (presence or absence of bands) were observed in HBP bands of the 30 bull capacitated frozen-thawed sperm extracts. Moreover, the inherent character of the proteins may also contribute toward the difference in number of bands. The findings are in accordance with the observations of Arangasamy [10] and Singh et al. [11] who reported eight (13, 14, 16, 20, 36, 41, 56 and 71 kDa) and nine HBP (14, 15, 20, 24, 33, 40, 55, 70 and 100 kDa), respectively in buffalo seminal plasma. Studies using one dimensional polyacrylamide gel

electrophoresis detected 35 bands of HBP in seminal plasma of rams and was associated with the sperm capacitation process [12]. Further, Moura et al. [13] detected 52 spots in accessory sex gland fluid of Holstein bulls by 2-dimensional electrophoresis indicating qualitative differences in HBP bands of all the bulls.

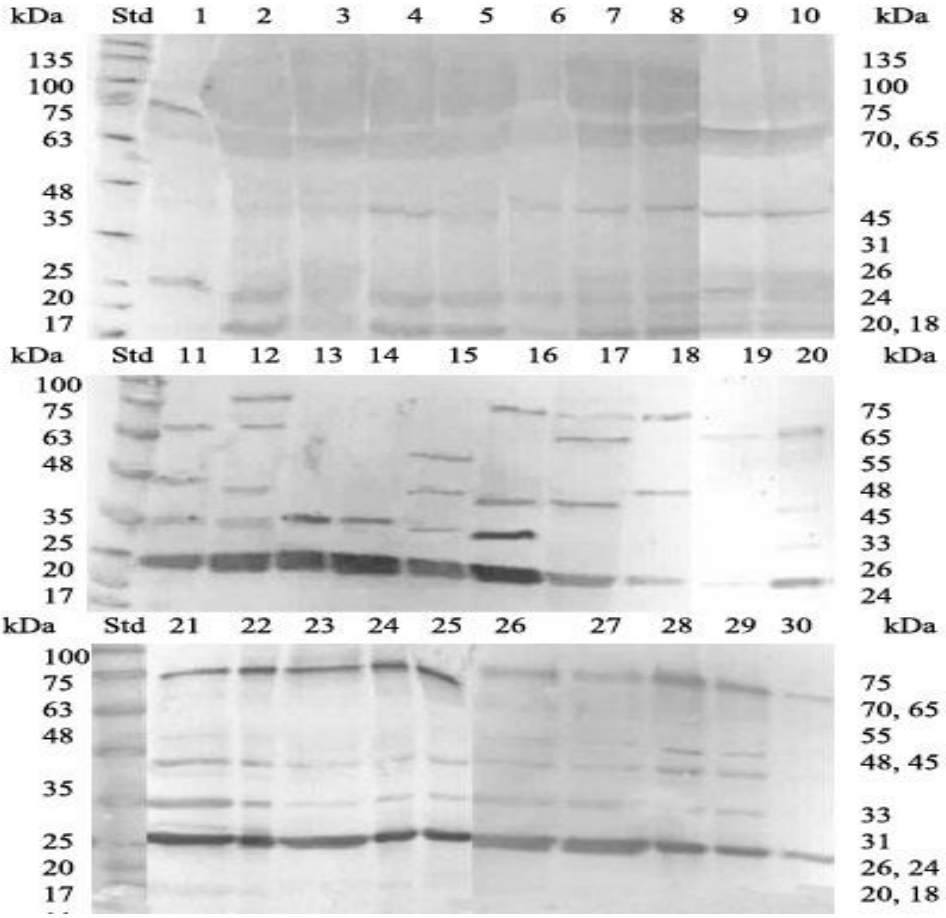

Figure 1: *Immunoblotting Pattern of HBP in Frozen-Thawed Capacitated/Acrosome-Reacted Sperm Extracts of Buffalo Bulls. Lane Std: Standard Protein Marker; Lanes 1-30: Bull Numbers*

3.2. Relationship of HBP differences with Acrosome Reaction and Fertility in Frozen-thawed Capacitated Sperm Extracts

The FSCR of bulls with HBP of 70, 31, 20 and 18 kDa was significantly ($P < 0.05$) higher than their counterparts. Although non-significant ($P > 0.05$), the FSCR was also higher in bulls positive for 135 and 100 kDa HBP as compared to their negative contemporary mates. An increase of 7.4, 7.4, 9.4, 10.9, 24.8 and 11.0% in the FSCR was recorded in the bulls with 135, 100, 70, 31, 20 and 18 kDa HBP, respectively as compared to their counterparts. The percentage of bulls with $\geq$ 50.0% FSCR was also higher (50.0, 50.0, 46.2, 62.5, 66.7 and 50.0%) amongst those positive for 135, 100, 70, 31, 20 and 18 kDa HBP than their respective counter mates (25.0, 25.0, 23.5, 22.7, 29.6 and 18.8%). No significant ($P > 0.05$) difference in the rate of acrosome reaction was observed between the bulls positive and negative for a 20 kDa HBP whereas, a significantly ($P < 0.05$) higher rate of acrosome reaction was recorded in bulls with HBP of 135, 100, 70 and 18 kDa in comparison to their contemporary mates with a difference of 7.4, 7.4, 8.7 and 9.4%, respectively. The FSCR of bulls with 75 kDa ($P < 0.05$), 55 kDa ($P < 0.05$) and 48 kDa ($P > 0.05$) was lower than their corresponding counterparts and had a decrease of 11.0, 16.7 and 11.0%, respectively. In addition, the proportion of bulls with good fertility was lower in the bull's positive as compared to negative ones (15.4, 0.0 and

0.0% vs 61.5, 38.7 and 38.7%, respectively). Likewise, the rate of acrosome reaction of bulls with HBP of 75, 55 and 48 kDa was significantly lower ($P < 0.05$) than their negative contemporary mates and a respective reduction of 7.3, 14.7 and 11.7% was observed. Similar studies [14] in buffalo bulls showed that heparin is the most potent enhancer of capacitation in buffalo spermatozoa. Further, induction of sperm capacitation in female reproductive tract is aided by HBP secreted by male accessory sex glands [15]. The HBP in seminal fluid attach themselves to the sperm surface, especially lipids containing the phosphoryl-choline group, thus allowing heparin-like glycosaminoglycans in female reproductive tract to activate sperm capacitation [4]. Therefore, seminal fluid HBP play a vital role in spermatozoon survival and the overall fertilization process, and any alteration in these proteins can directly be related to infertility.

Table 1: *Relationship of HBP with FSCR and Acrosome Reaction in Frozen-Thawed Capacitated Semen of Buffalo Bulls (Mean ± SE)*

Mol. Wt. (kDa)	Bulls positive for HBP			Bulls negative for HBP		
	Overall FSCR (%)	Percentage of bulls with ≥ 50.0% FSCR	Percent acrosome-reacted spermatozoa	Overall FSCR (%)	Percentage of bulls with ≥ 50.0% FSCR	Percent acrosome-reacted spermatozoa
135	40.9 ± 6.9	50.0 (5)$^{\#}$	56.7 ± 2.5^{c}	33.5 ± 3.4	25.0 (5)$^{\#}$	49.3 ± 2.3^{d}
100	40.9 ± 6.9	50.0 (5)$^{\#}$	56.7 ± 2.5^{c}	33.5 ± 3.4	25.0 (5)$^{\#}$	49.3 ± 2.3^{d}
75	30.8 ± 4.0^{a}	15.4 (2)$^{\#}$	47.6 ± 2.9^{c}	41.8 ± 4.5^{b}	47.1 (8)$^{\#}$	54.9 ± 2.1^{d}
70	42.3 ± 5.1^{a}	46.2 (6)$^{\#}$	56.7 ± 2.0^{c}	32.9 ± 3.9^{b}	23.5 (4)$^{\#}$	48.0 ± 2.5^{d}
65	37.2 ± 4.5	29.4 (5)$^{\#}$	51.7 ± 2.4	37.7 ± 4.4	38.5 (5)$^{\#}$	51.8 ± 2.9
55	22.5 ± 6.3^{a}	0.0 (0)$^{\#}$	39.3 ± 2.5^{c}	39.2 ± 3.4^{b}	38.5 (10)$^{\#}$	53.9 ± 1.7^{d}
48	27.5 ± 7.5	0.0 (0)$^{\#}$	41.6 ± 3.9^{c}	38.5 ± 3.4	38.5 (10)$^{\#}$	53.3 ± 1.9^{d}
45	37.8 ± 3.7	34.8 (8)$^{\#}$	52.7 ± 2.1	34.3 ± 6.5	28.6 (2)$^{\#}$	48.7 ± 3.8
33	36.4 ± 4.4	25.0 (4)$^{\#}$	49.9 ± 2.9	37.5 ± 4.7	42.9 (6)$^{\#}$	53.3 ± 2.3
31	45.0 ± 6.5^{a}	62.5 (5)$^{\#}$	56.1 ± 3.3	34.1 ± 3.5^{b}	22.7 (5)$^{\#}$	50.9 ± 2.2
26	38.0 ± 7.3	40.0 (2)$^{\#}$	56.7 ± 4.3	36.8 ± 3.6	32.0 (8)$^{\#}$	50.7 ± 2.0
24	36.2 ± 3.5	30.8 (8)$^{\#}$	51.0 ± 1.9	42.5 ± 7.5	50.0 (2)$^{\#}$	56.8 ± 5.5
20	60.0 ± 10.0^{a}	66.7 (2)$^{\#}$	49.8 ± 5.1	35.2 ± 3.3^{b}	29.6 (8)$^{\#}$	51.9 ± 2.0
18	42.9 ± 5.1^{a}	50.0 (7)$^{\#}$	56.7 ± 2.3^{c}	31.9 ± 3.7^{b}	18.8 (3)$^{\#}$	47.3 ± 2.3^{d}

Superscripts ^{a,b}differ significantly ($P < 0.05$) in same row for overall FSCR.
Superscripts ^{c,d}differ significantly ($P < 0.05$) in same row for acrosome-reacted spermatozoa.
Figures in parentheses with $^{\#}$ indicate number of tested bulls with ≥ 50.0% FSCR.

4. Conclusion

In conclusion, immunoblotting demonstrated that HBP are present on surface of capacitated spermatozoa and may play a significant role in regulating reproductive potential of buffalo bull semen. The HBP of 135, 100, 70 and 18 kDa did seem to activate the *in vitro* sperm acrosome reaction vis-à-vis higher conception rate in bulls' positive for these proteins as compared to their negative counterparts. More studies are clearly required to validate the functional relationship between presence of HBP on capacitated sperm and fertility of buffalo bulls.

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Effect of Various Ovulation Synchronization Protocols on Estrus Response, Conception Rate and Blood Biochemical Profile in Anoestrus Buffaloes

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Abstract This study evaluated the fertility response and plasma profile of biochemical and mineral constituents in 46 postpartum anoestrus (>90 days) buffaloes treated under field conditions with TriU-B/PRID, Ovsynch and Heatsynch protocols (n=12 each), keeping untreated anoestrus control (n=10) and normal cyclic control (n=10) groups. The estrus induction response with 3 treatment protocols was 83.33, 83.33 and 91.66 % respectively within 2-3 days of $PGF_2\alpha$ injection. The conception rates (CRs) obtained at induced estrus in buffaloes under TriU-B, Ovsynch and Heatsynch protocols were 25.00, 33.33 and 25.00 %, and those of overall three cycles' 50.00, 58.33 and 50.00 %, respectively, which were at par with normal cyclic control group (CR at first cycle 30.00 & overall of 3 cycles 50.00 %). Among untreated anoestrus control group, only two buffaloes (20%) exhibited estrus and conceived over 90 days follow up. The overall pooled mean plasma total cholesterol concentrations (mg/dl) in anoestrus buffaloes under TriU-B and in normal cyclic control group were the same (166.70±5.28 and 165.18±6.37) and significantly (P<0.01) higher than in Ovsynch (131.54±3.71) and lower than in Heatsynch (186.14±5.69) group. The value for total protein (g/dl) was significantly (P<0.05) lower in TriU-B (7.27±0.08) followed by other two groups (7.62±0.07 & 7.44±0.07) and highest in normal control group (7.98±0.10). The calcium was significantly higher (P<0.05) in Ovsynch (10.41±0.07 mg/dl) than other 3 groups (8.82±0.11 to 9.09±0.09 mg/dl), while inorganic phosphorus (3.99±0.10 to 4.29±0.13 mg/dl) did not vary between groups. Normal cyclic control buffaloes had significantly higher protein and lower calcium levels than in most treatment groups. The conceived buffaloes had apparently higher values of total cholesterol and protein as compared to non-conceived ones in all groups. In general, Ovsynch protocol was the best followed by TriU-B and Heatsynch for induction of estrus and improvement of fertility in anoestrus buffaloes.
Keywords *Anoestrus Buffalo; Estrus Synchronization Protocols; Blood Biochemical Profile; Fertility*

1. Introduction

In the recent years, considerable attention has been focused on reproductive endocrinology and blood biochemical and mineral profile as a means to identify specific reproductive and nutritional

problems and thereby to adopt appropriate therapeutic measures to augment fertility. Biochemical constituents of blood have great diagnostic value in evaluating the physiological status as well as in the clinical practice to improve postpartum fertility in female bovines. Use of hormonal protocols like TriU-B, Ovsynch and Heatsynch induces and synchronizes the estrus/ovulation and thus improves the conception rates and establishes cyclicity in acyclic buffaloes, thereby achieving ideal calving interval. Cholesterol is synthesized from acetate with a series of intermediate substances. It is an essential precursor for steroid hormone synthesis of testis, ovary and adrenal cortex. The thyroid hormones and estrogen influence cholesterol synthesis. Lack of protein or insufficient intake of protein was considered to be a cause of failure or delay in estrous cycle (Roberts, 1971). Protein deficiency is associated with retardation of the development of sex organs and may affect subsequently the reproductive performance (Herrick, 1977). Minerals like calcium, phosphorus and magnesium also influence the ability of animals to utilize other micro-minerals. The influence of these minerals on certain enzyme system may affect reproductive efficiency (Dhoble and Gupta, 1986), which might be reflected in lower blood level of them. Lack of minerals especially calcium and phosphorus upsets the proper functioning of the genital organs (Acharya, 1960). Hence, this study was planned to evaluate the comparative efficacy of TriU-B, Ovsynch and Heatsynch protocols for fertility enhancement and to see their influence on plasma biochemical and mineral profile in anoestrus rural buffaloes.

2. Materials and Methods

The present investigation was carried out under field conditions of Anand and Mahisagar districts of middle Gujarat and at Livestock Research Station, NAU, Navsari, South Gujarat. A total of 46 postpartum true anoestrus (>90 days) buffaloes and 10 normal cyclic control buffaloes that exhibited spontaneous estrus within 90 days postpartum constituted the experimental animals. All the infertile animals identified were dewormed using Inj. Ivermectin @ 100 mg s/c and were treated initially once with i/m injection of inorganic phosphorus (Inj. Alphos-40 @ 10 ml, Pfizer) and multivitamins AD_3E (Inj. Vetacept @ 10 ml, Concept Pharma), and oral multi-minerals (Minotas, Intas Pharma) @ 1 bolus daily for 7 days. The anoestrus buffaloes were then randomly subjected to different standard estrus induction/synchronization protocols (TriU-B, Ovsynch and Heatsynch, n=12 each, Buhecha et al., 2016). Another 10 anoestrus animals were kept as anoestrus control and 10 normal cyclic buffaloes served as normal cyclic control group. Buffaloes in spontaneous or induced estrus were inseminated using good quality frozen-thawed semen. Buffaloes detected in estrus subsequent to FTAI were re-inseminated up to 2 cycles and in non-return cases pregnancy was confirmed per rectum 60 days of last AI.

Jugular blood samples were collected four times in heparinized vaccutainers from true anoestrus animals, i.e. on day 0 - just before treatment, on day 7 - at the time of PGF2α administration, on day 9/10 - induced estrus/FTAI and on day 21 post-AI. Blood sampling for two control groups of animals was done on the day of spontaneous estrus if any, and on day 21 post-AI. The plasma separated out by centrifugation of blood at 3000 rpm for 15 min was stored at -20°C with a drop of merthiolate (0.1%) until analyzed. Plasma total protein, cholesterol, calcium and inorganic phosphorus were estimated by using standard procedures and assay kits (Analytical Technologies Pvt. Limited, Baroda) on biochemistry analyzer. The Chi square test was used to compare conception rate while plasma profile of biochemical and mineral constituents was analyzed using one way analysis of variance and ‘t’ test (Snedecor and Cochran, 1994) using online SAS software version 20.00.

3. Results and Discussion

3.1. Estrus Induction and Fertility Response to Synchronization Protocols

In anoestrus buffaloes under TriU-B, Ovsynch and Heatsynch protocols, estrus induction response was 83.33, 83.33 and 91.66 % within mean intervals of 69.30±0.80, 70.60±1.30 and 69.20±1.49 h, from PG injection, with prominent to moderate estrus signs. The conception rates obtained at induced estrus in three protocols were 25.00, 33.33, and 25.00 %, and the overall conception rates of three cycles were 50.00, 58.33 and 50.00 %, respectively. Moreover, by 60 days of post-induction/AI in TriU-B, Ovsynch and Heatsynch protocols among treated non-conceived buffaloes 2 (16.66%), 3 (24.99%) and 4 (33.33%) buffaloes turned out to be anoestrus. In untreated anoestrus control group, out of 10 buffaloes only 2 buffaloes (20 %) expressed spontaneous estrus after 41 and 63 days of initiation of experiment and conceived giving overall conception rate of 20.00 %. In normal cyclic control group, the conception rate at first and overall of 3 cycles was 30.00 and 50.00 %, respectively.

The present findings of estrus synchronization rates with TriU-B (simulating CIDR) and Ovsynch were in accordance with the earlier results with CIDR and Ovsynch protocols by Campenile et al. (2005), Ali et al. (2012) and Kundalkar et al. (2014) in different breeds of buffaloes. TriU-B being newly launched product, no report on its use could be seen in the literature, except one of our own (Buhecha et al., 2016). Similarly, it was difficult to find report on use of Heatsynch protocol in buffaloes, except that of Mirmahmoudi et al. (2014), wherein the estrus induction response was relatively poor in both cyclic and acyclic buffaloes. The comparable conception rates at induced estrus and overall of three cycles in Ovsynch and CIDR protocols have been documented by Baruselli et al. (2003) as 52.50 and 28.20 %, Naikoo et al. (2010) as 50.00 and 50.00 % and Ali et al. (2012) as 60.00 and 33.33 %, respectively. However, relatively better results than the present ones were found earlier using similar protocols in buffaloes from Gujarat climate by Naikoo et al. (2010), Savalia et al. (2014) and Nakrani et al. (2014).

3.2. Plasma Total Protein and Total Cholesterol

The mean levels of plasma total cholesterol recorded on day 0, 7, 9 (AI) of treatment and on day 21 post-AI in buffaloes under TriU-B, Ovsynch and Heatsynch protocols (Table 1) did not reveal significant differences in the profile between days/periods of the treatment in any of the groups, but it varied significantly between the groups, with inconsistent trend. Similar results were observed in anoestrus buffaloes under CIDR and Ovsynch protocols by Savalia et al. (2014). Parmar (2013) and Nakrani et al. (2014) reported that the influence of periods (day 0, 7, 9 of treatment and day 21 post-AI) and treatment groups (CIDR, Ovsynch and Crester protocols) in anoestrus buffaloes was not significant for the plasma total cholesterol profile. This may be due to difference in the nutritional status of animals and the assay kits and procedures followed. In the present study, at day 21 post-AI there were insignificantly low mean plasma total cholesterol levels in conceived as compared to non-conceived buffaloes. This trend was in line with the earlier observations of Savalia et al. (2014) and Nakrani et al. (2014), though their values were much lower than the present findings. Apparently or significantly lower plasma total cholesterol profile noted in anoestrus animals in some of the reports prove that the steroid hormone precursor cholesterol was not available in sufficient quantity to synthesize estrogen in the growing follicles triggering ovarian cyclicity and estrus in such animals.

Table 1: *Plasma Total Cholesterol Concentrations (mg/dl) in Anoestrus Buffaloes on Different Days of Various Estrus Synchronization Treatments*

Estrus Induction Protocol	Status	No.	Plasma Total Cholesterol Concentrations (mg/dl)				Overall
			D-0	D-7	D-9/10 (AI)	D-21 post-AI	
TriU-B	Conceived	3	149.65 ± 15.73	153.18 ± 10.61	150.11 ± 14.04	149.96 ± 19.64^{p}	150.73 ± 6.88^{p}
	Non-conc.	5	174.19 ± 12.02	176.61 ± 13.15	188.69 ± 13.28	191.23 ± 10.56^{q}	182.68 ± 5.82^{q}
	Overall	**8**	**161.92 ± 10.27**	**164.90 ± 8.99**	**169.40 ± 11.54**	**170.60 ± 12.94**	**166.70 ± 5.28^{y}**
Ovsynch	Conceived	4	143.32 ± 7.07	135.69 ± 10.50	123.83 ± 12.88	121.08 ± 10.24	130.98 ± 5.19
	Non-conc.	4	125.66 ± 8.18	143.18 ± 21.37	130.77 ± 2.70	128.79 ± 2.72	132.10 ± 5.46
	Overall	**8**	**134.49 ± 6.02**	**139.44 ± 11.11**	**127.30 ± 6.23**	**124.93 ± 5.12**	**131.54 ± 3.71^{x}**
Heatsynch	Conceived	3	185.18 ± 2.82	197.25 ± 4.24	196.67 ± 11.43	183.61 ± 3.56	190.68 ± 3.36
	Non-conc.	5	181.76 ± 20.49	167.85 ± 19.07	190.92 ± 20.64	193.16 ± 14.27	183.42 ± 8.92
	Overall	**8**	**183.04 ± 12.30**	**178.87 ± 12.68**	**193.08 ± 12.93**	**189.58 ± 8.78**	**186.14 ± 5.69^{z}**
Normal Cyclic Control	Conceived	3	-	-	151.27 ± 6.81	149.51 ± 9.28	150.38 ± 5.16
	Non-conc.	5	-	-	174.34 ± 9.33	173.77 ± 15.99	174.05 ± 8.72
	Overall	**8**	-	-	**165.69 ± 7.34**	**164.67 ± 10.97**	**165.18 ± 6.37^{y}**

Means bearing uncommon superscripts within column (x, y) and subgroup (p, q) differ significantly (P < 0.05).
D-0 = Day of starting the treatment, D-7 = Administration of PG, D-9/10 = Fixed time AI, D-21= Day 21 post-AI.

The mean plasma levels of total proteins recorded on different days of treatment and on day 21 post-AI in buffaloes under three treatment protocols (Table 2) revealed that the profile did not vary significantly between sampling days in any of the protocols/groups. Savalia et al. (2014) and Nakrani et al. (2014) also reported similar non-significant variations in the overall mean plasma protein concentrations in buffaloes under CIDR, Ovsynch and Crester protocols and between days of sampling. However, the overall mean concentrations were significantly (P<0.05) higher in conceived than non-conceived buffaloes in TriU-B and in normal cyclic control and insignificantly higher in Ovsynch and Heatsynch groups. Similar trend was also found by Savalia et al. (2014) and Nakrani et al. (2014). The results of the present study proved that the plasma total protein profile is not influenced significantly by the hormonal treatments used in dairy animals, and that higher plasma protein levels are indicative of better nutritional status/ nitrogen balance, which favours the sound reproductive performance in animals.

Table 2: *Plasma Total Protein Concentrations (g/dl) in Anoestrus Buffaloes on Different Days of Various Estrus Synchronization Treatments*

Estrus Induction Protocol	Status	No.	Plasma Total Protein Concentrations (g/dl)				Overall
			D-0	D-7	D-9/10 (AI)	D-21 post-AI	
TriU-B	Conceived	3	7.35 ± 0.14	7.25 ± 0.28	7.50 ± 0.32	7.66 ± 0.25	7.44 ± 0.12^{q}
	Non-conc.	5	7.17 ± 0.19	7.17 ± 0.24	7.01 ± 0.16	7.04 ± 0.10	7.10 ± 0.08^{p}
	Overall	**8**	**7.26 ± 0.11**	**7.21 ± 0.17**	**7.25 ± 0.19**	**7.35 ± 0.17**	**7.27 ± 0.08^{x}**
Ovsynch	Conceived	4	7.65 ± 0.18	7.51 ± 0.15	7.62 ± 0.22	7.66 ± 0.25	7.65 ± 0.09
	Non-conc.	4	7.67 ± 0.18	7.47 ± 0.27	7.85 ± 0.25	7.56 ± 0.25	7.61 ± 0.11
	Overall	**8**	**7.66 ± 0.12**	**7.49 ± 0.14**	**7.74 ± 0.16**	**7.61 ± 0.16**	**7.62 ± 0.07^{y}**
Heatsynch	Conceived	3	7.20 ± 0.24	7.13 ± 0.22	7.20 ± 0.22	7.65 ± 0.16	7.54 ± 0.11
	Non-conc.	5	7.64 ± 0.06	7.62 ± 0.26	7.50 ± 0.17	7.41 ± 0.17	7.45 ± 0.08
	Overall	**8**	**7.47 ± 0.12**	**7.44 ± 0.19**	**7.39 ± 0.13**	**7.50 ± 0.17**	**7.44 ± 0.07^{xy}**
Normal Cyclic Control	Conceived	3	-	-	8.25 ± 0.11	8.39 ± 0.10	8.32 ± 0.07^{q}
	Non-conc.	5	-	-	7.72 ± 0.19	7.83 ± 0.16	7.77 ± 0.12^{p}
	Overall	**8**	-	-	**7.92 ± 0.15**	**8.04 ± 0.15**	**7.98 ± 0.10^{z}**

Means bearing uncommon superscripts within column (x, y, z) and subgroup (p, q) differ significantly (P<0.05).
D-0 = Day of starting the treatment, D-7 = Administration of PG, D-9/10 = Fixed time AI, D-21 = Day 21 post-AI.

3.3. Plasma Calcium and Phosphorus

The results on levels of plasma calcium and phosphorus concentrations obtained on day 0, 7, 9/10 (AI) of treatment and on day 21 post-AI in buffaloes under TriU-B, Ovsynch and Heatsynch protocols, and on day of AI and day 21 post-AI in normal cyclic group are presented in Table 3.

Table 3: *Plasma Calcium and Inorganic Phosphorus Concentrations (mg/dl) in Anoestrus Buffaloes on Different Days of Various Estrus Synchronization Treatments*

Blood Parameter	Estrus Induction Protocol	No.	Plasma Calcium and Phosphorus Concentrations (mg/dl)				Overall
			D-0	D-7	D-9/10 (AI)	D-21 post-AI	
Plasma Calcium	TriU-B	8	9.12 ± 0.26	9.02 ± 0.30	9.04 ± 0.23	9.06 ± 0.27	9.06 ± 0.13^{x}
	Ovsynch	8	10.33 ± 0.08	10.32 ± 0.18	10.53 ± 0.11	10.47 ± 0.17	10.41 ± 0.07^{y}
	Heatsynch	8	9.06 ± 0.20	8.98 ± 0.24	9.07 ± 0.15	9.26 ± 0.15	9.09 ± 0.09^{x}
	Normal Cyclic	8	-	-	8.73 ± 0.15	8.92 ± 0.18	8.82 ± 0.11^{x}
Plasma Inorganic Phosphorus	TriU-B	8	3.81 ± 0.21	4.01 ± 0.25	4.20 ± 0.28	4.10 ± 0.13	4.03 ± 0.13
	Ovsynch	8	4.32 ± 0.31	4.25 ± 0.22	4.22 ± 0.27	4.37 ± 0.28	4.29 ± 0.13
	Heatsynch	8	3.98 ± 0.23	3.93 ± 0.23	3.85 ± 0.26	4.21 ± 0.10	3.99 ± 0.10
	Normal Cyclic	8	-	-	4.00 ± 0.17	4.21 ± 0.19	4.10 ± 0.12

Means bearing uncommon superscripts within column (x, y) for a trait differ significantly (P < 0.05). D-0 = Day of starting the treatment, D-7 = Administration of PG, D-9/10 = Fixed time AI, D-21 = Day 21 post-AI.

These results did not reveal significant variations in plasma calcium concentrations of buffaloes between days/periods of any treatment and in normal cyclic control group. Similarly the variations in plasma calcium levels between conceived and non-conceived groups were not significant, though the levels were apparently higher in conceived than non-conceived animals. Further, the overall mean values of plasma calcium obtained in anoestrus buffaloes under TriU-B and Heatsynch protocols and in normal cyclic buffaloes were 9.06±0.13, 9.09±0.09 and 8.82±0.11 mg/dl, respectively, which were statistically similar. However, the mean plasma calcium concentration noted in Ovsynch protocol (10.41±0.07 mg/dl) was significantly higher (P<0.05) as compared to the values of other treatment and control groups. Very similar observations have been recently documented by Savalia et al. (2013) and Nakrani et al. (2014)) using CIDR in buffaloes, including normal cyclic control group. Further, Savalia et al. (2013) also obtained higher mean calcium levels in conceived as compared to non-conceived buffaloes under CIDR and Ovsynch protocols.

Further, the plasma concentrations of inorganic phosphorus in different groups closely followed the plasma calcium profile. The levels neither varied significantly between sampling days/periods in any of the treatment protocols and control group, nor between conceived and non-conceived buffaloes in any of the groups. The overall mean values of inorganic phosphorus obtained in anoestrus buffaloes under TriU-B, Ovsynch, Heatsynch and cyclic buffaloes were 4.03±0.13, 4.29±0.13, 3.99±0.10 and 4.10±0.12 mg/dl, respectively, which did not differ significantly among themselves. Very similar observations have been recently made by some of the workers using CIDR and Ovsynch protocols in anoestrus buffaloes, including normal cyclic control groups (Savalia et al., 2013; Nakrani et al., 2014) from the same institute. Bhaskaran and Abdulla Khan (1981) documented that the marginal deficiency of phosphorus is enough to cause disturbances in pituitary-ovarian axis, without manifesting specific systemic deficiency symptoms. Butani et al. (2011), Parmar et al. (2012) and Savalia et al. (2013) did not find appreciable variation in the mean plasma inorganic phosphorus levels on the day of GnRH and/or PG treatment, at induced oestrus and on day 21 post-AI in anoestrus or suboestrus buffaloes.

It was concluded that all the three protocols, viz, TriU-B, Ovsynch and Heatsynch used in true anoestrus buffaloes resulted into more than 83 % estrus induction with establishment of cyclicity and

good conception rates (50 to 58 %) without significantly altering the plasma biochemical and minerals profile suggestive of better scope for their use under field conditions.

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Effect of Ovsynch and Mid-Cycle $PGF_2\alpha$ Treatment Protocols on Conception Rates and Plasma Biochemical and Minerals Profile in Repeat Breeding Cows and Buffaloes

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Abstract This study was conducted to evaluate the efficacy of Ovsynch and Mid-cycle $PGF_2\alpha$ protocols on conception rate and influence on plasma biochemical and mineral constituents in 20 each repeat breeding cows and buffaloes equally divided into Ovsynch and Mid-cycle $PGF_2\alpha$ protocols. Ten each healthy early postpartum cows and buffaloes served as normal cyclic controls. All the animals were inseminated at mid-estrus using good quality frozen-thawed semen and were kept under observation for next estrus or pregnancy. The first service conception rates at induced estrus obtained in repeat breeding cows and buffaloes were 50.00 and 40.00 % in Ovsynch protocol, and 40.00 and 50.00 % in mid-cycle $PGF_2\alpha$ treatment, respectively, and 40.00 % each in normal control group. The overall mean values of total protein for conceived and non-conceived cows of Ovsynch protocol were 7.98±0.17 and 7.38±0.11 g/dl and for buffaloes 9.18±0.18 and 7.30±0.05 g/dl, respectively, being significantly higher in conceived ones in both the species. The mean cholesterol level in Ovsynch protocol was insignificantly higher in non-conceived than conceived cows (197.43±13.45 vs. 176.80±9.54 mg/dl). Contrary to this finding, in buffaloes it was higher in conceived than non-conceived group (149.43±9.02 vs. 135.65±9.81 mg/dl). No significant difference was observed in total protein and cholesterol profile between conceived and non-conceived groups of cows and buffaloes under Mid-cycle PG treatment. Also no significant differences were observed in values of calcium and inorganic phosphorus between days and between conceived and non-conceived groups of cows or buffaloes, apparently the values of both were higher in normal cyclic groups as compared to repeat breeders. The overall pooled values of calcium and phosphorus in cows under Ovsynch protocol were 9.17±0.12 and 4.44±0.10, and under Mid-cycle $PGF_2\alpha$ group 9.23±0.10 and 4.61±0.05 mg/dl, respectively, while in buffaloes the corresponding values under Ovsynch protocol were 9.88±0.10 and 4.94±0.05, and under Mid-cycle $PGF_2\alpha$ group 9.33±0.10 and 4.67±0.05 mg/dl, respectively. From the results, it can be inferred that hormonal therapies used significantly improved the pregnancy rates in repeat breeding cows and buffaloes under field conditions, without influencing the plasma biochemical and minerals profiles.

Keywords *Repeat Breeding Bovines; Ovsynch; Mid-Cycle PGF2α Treatment; Conception Rate; Biochemical & Mineral Profile*

1. Introduction

Numerous studies have shown that repeat breeding is the second most prevalent reproduction disorder after anoestrus in dairy cows and buffaloes with the overall prevalence as high as 28.31 % (Bhat et al., 2012). Studies have shown that Ovsynch programme, with a strategic combination of GnRH (two injections) and $PGF_2\alpha$ (one injection), result in an acceptable pregnancy rate in cyclic cows (Pursley et al., 1995) and buffaloes (Baruselli et al., 1999). The recent reports demonstrated that the Ovsynch protocol resulted in increased conception rates by 21 per cent in repeat breeder dairy cows (Kasimanickam et al., 2005) and mid cycle $PGF_2\alpha$ treatment gave conception rate up to 70 per cent (Patel et al., 2014[a]) without influencing the plasma biochemical and mineral profile. In view of the above fact, the present study was aimed to study the relative efficacies of two hormonal approaches in repeat breeding crossbred cows and buffaloes in terms of estrus response, conception rate and plasma biochemical and mineral profile.

2. Materials and Methods

2.1. Selection and Treatment of Animals

This study was conducted from November 2014 to April 2015 on repeat breeding dairy animals selected from the villages of Amul as well as Panchamrut milk shed areas of Gujarat and at Livestock Research Station, NAU, Navsari. Per rectum palpation of the reproductive tract was conducted in specific animals, which had been bred more than three times yet not conceived, to diagnose them as classical repeat breeders. Twenty each repeat breeding cows and buffaloes and 10 each normal cyclic early postpartum cows and buffaloes were selected for this study.

All animals identified were treated with s/c injection of ivermectin 100 mg (Inj. Neomec, 1 %, 10 ml, Intas Pharmaceuticals Pvt Ltd., Ahmedabad), i/m injections of inorganic phosphorus (Inj. Alphos-40, 10 ml, Zoetis), multivitamins AD3E (Inj. Intavita, 10 ml, Intas), and single shot enrofloxacin 3.0 g (Inj. Flobac SA, 10 %, 30 ml, Intas), and were supplied with four multi-mineral boli (Garbhamin, Indian Immunologicals Ltd.) for PO use, one bolus on alternate day. The repeat breeder cows and buffaloes were then randomly allotted to the following treatment protocols.

2.2. Ovsynch Protocol (Group-I)

Ten repeat breeding cows and buffaloes each with normal sized ovaries were put under Ovsynch protocol and were administered with i/m Inj. of Buserelin acetate 10 µg (Ovulanta, 2.5 ml, Vet Mankind) on day 0, Inj. $PGF_2\alpha$ 500 µg (Repregna, 2 ml, Vet Mankind) on day 7, and second i/m Inj. of Buserelin acetate was given on day 9, and fixed time AI (FTAI) was performed twice at 0 and 24 hrs later.

2.3. Mid-cycle PGF2α Treatment (Group-II)

Another ten repeat breeding cows and buffaloes each having mid-cycle palpable CL were put under Mid-cycle $PGF_2\alpha$ treatment and were given i/m Inj. of $PGF_2\alpha$ 500 µg (Repregna, 2 ml) and FTAIs were performed twice at 72 and 96 hrs later.

2.4. Normal Cyclic Control (Group-III)

Ten each normal cyclic cows and buffaloes exhibiting estrus within 90 days postpartum and bred without any treatment served as control.

2.5. Blood Sampling for Plasma Profile

Jugular blood samples were collected from 8 animals in heparinized vacutainers on day 0 (day of first GnRH inj.), day 7 (day of $PGF_2\alpha$ injection), day 9 (day of second GnRH inj/AI) of treatment and on day 21 post-AI in Ovsynch group, and on day of $PGF_2\alpha$ injection, day of induced estrus/AI and day 21 post-AI in Mid-cycle $PGF_2\alpha$ group, while in control group blood samples were obtained on day of spontaneous estrus/AI and 21 days post-AI. The blood samples were centrifuged and plasma stored at -20°C in deep freezer. The estimations of plasma protein, cholesterol, calcium and phosphorus were done using standard procedures and assay kits of Crest Biosystem, Goa on semi-autoanalyzer.

2.6. Statistical Analysis

The conception rates in different groups were compared by Chi-square test. The data on plasma biochemical and mineral profile were analyzed using one way analysis of variance and 't' test to compare variation within and between group (Snedecor and Cochran, 1994).

3. Results and Discussion

3.1. Effect of Hormone Protocols on Fertility

All the repeat breeding cows and buffaloes (100 %) under both Ovsynch and mid-cycle $PGF_2\alpha$ treatment protocol exhibited induced estrus within 2 to 4 days from day of $PGF_2\alpha$ injection. These observations are in line with the response and intervals reported earlier by Sathiamoorthy et al. (2007), Khasatiya et al. (2008), Naikoo et al. (2010), Patel et al. (2014[a]) and Nakrani et al. (2015) in cows and buffaloes.

The conception rates at induced (first) estrus following Ovsynch protocol in cows and buffaloes were 50.00 and 40.00 %, with some 20 to 33 % animals conceiving with 2nd and 3rd cycle post-treatment, thus giving the overall pregnancy rates of three cycles as 70.00 and 60.00 %, respectively. The conception rates in Mid-cycle PGF2α treated repeat breeder cows and buffaloes were found to be 40.00 and 50.00 % at induced estrus, with some 16.66 to 25.00 % animals conceiving in 2nd and 3rd estrus, with the overall pregnancy rates of 60.00 and 70.00 %, respectively. In normal cyclic control cows and buffaloes also the conception rates at first AI were 40.00 and 40.00 % and those of overall of three cycles 70.00 and 60.00 %, respectively. The conception rates in treated cows and buffaloes improved significantly and were statistically at par with control groups. These findings of conception rates are in accordance with the earlier reports of 61 to 82 % by Tenhagen et al. (2004), Ali and Fahmy (2007), Biradar et al. (2014) and Nakrani et al. (2015). However, Paul and Prakash (2005), Karen and Darwish (2010) and Derar et al. (2012) recorded lower conception rates of 33.33, 18.00 and 22.71%, respectively, with Ovsynch protocol. The first service conception rates of 40-50% found for mid-cycle $PGF_2\alpha$ treated repeat breeders are little better than the reports of Sathiamoorthy et al. (2007) and Savalia et al. (2014) in buffaloes and Patel et al. (2014[a]) in crossbred cows. The present finding of 60.00 % overall pregnancy rate found in Mid-cycle PGF2α treated repeat breeder cows is similar with the conception rate recorded by Patel et al. (2005). The pregnancy rate of the three services found as 70.00 % following $PGF_2\alpha$ induced estrus in buffaloes is however lower than the pregnancy rates reported by Dhami et al. (2009, 2014) and Khasatiya et al. (2008).

3.2. Plasma Protein and Cholesterol Profile

The mean total protein concentration was relatively lower in conceived than non-conceived cows under Ovsynch protocol (7.65±0.12 g/dl vs. 8.06±0.26 g/dl), while relatively higher level was found in buffaloes for the same protocol (9.41±0.05 g/dl vs. 9.27±0.10 g/dl). Also mean total protein concentrations of Mid-cycle $PGF_2\alpha$ inj. group in cows (7.39±0.12 g/dl) and buffaloes (7.32±0.07 g/dl)

were slightly higher in conceived than the non-conceived groups (7.28±0.11 and 7.19±0.07, respectively). Repeat breeder buffaloes treated with various hormonal therapies had relatively higher values of plasma total protein compared with normal cyclic buffaloes. No significant differences were observed in plasma total protein profiles between periods of the treatment. Higher plasma protein concentration was observed in conceived than non-conceived groups of both cows and buffaloes under Ovsynch protocol, but the difference was significant only in cows (Table 1).

Table 1: *Mean Plasma Total Protein Levels (G/Dl) in Repeat Breeding Cows and Buffaloes under Ovsynch Protocol and Mid-Cycle $PGF_2\alpha$ Inj. on Different Days of Treatment/AI*

	Treatment Protocol	Status	No	Days from Treatment/AI				Overall
				D-0	D-7	D-AI	D-21	
Cow	Ovsynch	C	5	7.93±0.39	8.43±0.48	7.66±0.19	7.90±0.28	7.98^{q}±0.17
		NC	3	7.41±0.13	7.76±0.20	7.10±0.31	7.23±0.10	7.38^{p}±0.11
		Pooled	8	7.74±0.25	8.18±0.32	7.45±0.18	7.65±0.21	7.75^{yz}±0.13
	Mid Cycle $PGF_2\alpha$ inj.	C	4	7.54±0.17	--	7.38±0.23	7.26±0.25	7.39±0.12
		NC	4	7.26±0.27	--	7.40±0.17	7.19±0.12	7.28±0.11
		Pooled	8	7.40±0.16	--	7.39±0.13	7.22±0.13	7.34^{xy}±0.08
	Normal Cyclic Control	C	4	--	--	7.93±0.56	8.62±0.53	8.27±0.38
		NC	4	--	--	7.74±0.15	7.69±0.35	7.71±0.18
		Pooled	8	--	--	7.83±0.27	8.15±0.34	7.99^{z}±0.21
Buffalo	Ovsynch	C	4	8.83±0.52	8.82±0.38	9.51±0.16	9.55±0.03	9.18^{q}±0.18
		NC	4	7.26±0.11	7.45±0.04	7.27±0.10	7.23±0.11	7.30^{p}±0.05
		Pooled	8	8.05±0.39	8.13±0.31	8.39±0.43	8.39±0.44	8.24^{y}±0.19
	Mid Cycle $PGF_2\alpha$ inj.	C	5	7.27±0.16	--	7.38±0.09	7.31±0.11	7.32±0.07
		NC	3	7.36±0.17	--	7.12±0.11	7.11±0.08	7.19±0.07
		Pooled	8	7.30±0.11	--	7.28±0.08	7.23±0.08	7.27^{x}±0.05
	Normal Cyclic Control	C	4	--	--	7.12±0.06	7.30±0.07	7.21±0.05
		NC	4	--	--	7.06±0.07	7.16±0.05	7.11±0.04
		Pooled	8	--	--	7.09^{a}±0.04	7.23^{b}±0.05	7.16^{x}±0.04

C = conceived, NC = non-conceived; Day-0 = Day of treatment, D-AI = Day of AI, D-21 = Day 21 post-AI; Means bearing uncommon superscripts within the row (a,b) and column (p,q) (x,y) differ significantly (P<0.05).

Cetin et al. (2002) found relatively identical serum total protein levels at estrus in repeat breeder and fertile cows. Dhoble et al. (2004) found significantly higher levels of plasma total protein in pregnant (7.92 g/dl) than non-pregnant cows (7.71 g/dl), which is comparable with the present findings in cows and buffaloes in normal cyclic, Mid-cycle $PGF_2\alpha$ protocol, and Ovsynch protocol in buffaloes, while in cows under Ovsynch protocol, plasma total protein was lower in conceived than non-conceived animals. Parmar (2013) and Patel et al. (2014^{a}) reported much higher mean total protein concentration in non-conceived than conceived repeat breeder buffaloes (11.30±0.30 vs 11.18±0.40 g/dl) and cows (12.39±0.25 vs 12.16± 0.28 g/dl) treated with Mid-Cycle $PGF_2\alpha$ inj. as compare to the present findings.

Like protein, there were no significant differences in the plasma total cholesterol profiles between days/periods of the treatment with Ovsynch protocol or Mid-cycle $PGF_2\alpha$ inj. group and also between conceived and non-conceived cows and buffaloes of any group, but the mean values at day 21 post-AI in non-conceived cows in Ovsynch protocol (197.43±13.45 mg/dl), Mid-cycle $PGF_2\alpha$ inj. group (217.58±12.05 mg/dl), and in normal cyclic group (211.47±5.85 mg/dl) were apparently higher as compared to those obtained in conceived groups (176.80±9.54 mg/dl, 200.89±10.51 mg/dl, and 191.96±8.62 mg/dl, respectively; Table 2).

Table 2: *Mean Plasma Total Cholesterol Concentration (Mg/Dl) In Repeat Breeding Cows and Buffaloes under Ovsynch Protocol and Mid-Cycle $PGF_2\alpha$ Inj. on Different Days of Treatment/AI*

	Treatment Protocol	Status	No	Days from Treatment/AI				Overall
				D-0	D-7	D-AI	D-21	
Cow	Ovsynch	C	5	163.29±22.52	179.23±26.46	185.50±18.49	179.20±10.66	176.80±9.54
		NC	3	163.88±13.82	184.71±11.85	211.99±25.56	229.13±41.78	197.43±13.45
		Pooled	8	163.51±14.20	181.28±16.31	195.43±14.68	197.93±17.64	184.53^{x}±7.89
	Mid Cycle $PGF_2\alpha$ inj.	C	4	187.49±13.26	--	207.26±29.97	207.92±7.12	200.89±10.51
		NC	4	236.13±18.09	--	200.30±17.86	216.33±27.12	217.58±12.05
		Pooled	8	211.81±13.87	--	203.78±16.20	212.13±13.08	209.24^{xy}±8.01
	Normal Cyclic Control	C	4	--	--	192.88±12.77	191.04±13.53	191.96±8.62
		NC	4	--	--	217.99±7.19	204.95±8.92	211.47±5.85
		Pooled	8	--	--	205.44±8.28	197.99±7.95	201.71^{x}±5.63
Buffalo	Ovsynch	C	4	149.98±16.00	161.65±19.72	142.32±18.84	143.76±23.43	149.43±9.02
		NC	4	125.56±18.18	131.56±24.51	140.73±26.96	144.77±13.75	135.65±9.81
		Pooled	8	137.77±12.12	146.60±15.63	141.53±15.23	144.27±12.58	142.54^{xy}±6.67
	Mid Cycle $PGF_2\alpha$ inj.	C	5	143.12±14.36	--	148.69±9.41	129.54±1.98	140.45±5.75
		NC	3	154.27±6.13	--	143.79±8.62	148.72±4.36	148.93±3.63
		Pooled	8	147.30±9.04	--	146.86±6.36	136.73±3.97	143.63^{xy}±3.88
	Normal Cyclic Control	C	4	--	--	142.71±2.99	142.13±9.29	142.42±4.52
		NC	4	--	--	146.77±3.77	150.19±4.95	148.48±2.95
		Pooled	8	--	--	144.74±2.35	146.16±5.11	145.45^{y}±2.72

C = conceived, NC = non-conceived; Day-0 = Day of treatment, D-AI = Day of AI, D-21 = Day 21 post-AI; Means bearing uncommon superscripts within the row (a,b) and column (p,q) (x,y) differ significantly (P<0.05).

Noble et al. (1977) and Chandrakar et al. (2003) observed low plasma total cholesterol level during fertile estrus compared to infertile estrus in buffaloes and cows, which corroborated with the present findings. The findings documented by Cetin et al. (2002) were also very much similar to the present observations. Chaudhari (2012) observed no significant difference between conceived and non-conceived crossbred cows under Ovsynch as well as Mid-cycle $PGF_2\alpha$ protocols, which corroborated well with the present findings. Similarly, no significant difference was observed in total cholesterol concentrations between conceived and non-conceived repeat breeding cows and buffaloes under mid-cycle $PGF_2\alpha$ protocol by Parmar (2013), Patel et al. (2014^{a}). The present findings thus support the observations of previous researchers.

3.3. Plasma Calcium and Phosphorus Profile

The plasma calcium and inorganic phosphorus levels presented in Table 3 and 4 did not reveal significant variations between days/periods of the treatment with Ovsynch and Mid-cycle $PGF_2\alpha$ protocol and in normal cyclic groups and even between conceived and non-conceived groups. There was no significant difference between treatment groups and control group and also no significant difference was observed between cows and buffaloes.

Table 3: *Mean Plasma Calcium Levels (Mg/Dl) in Repeat Breeding Cows and Buffaloes under Ovsynch Protocol and Mid-Cycle $PGF_2\alpha$ Inj. on Different Days of Treatment/AI*

	Treatment Protocol	Status	No	Days from Treatment/AI				Overall
				D-0	D-7	D-AI	D-21	
Cow	Ovsynch	C	5	8.58±0.53	9.41±0.20	9.33±0.24	9.29±0.33	9.15±0.18
		NC	3	9.39±0.22	9.13±0.40	8.85±0.29	9.38±0.16	9.19±0.14
		Pooled	8	8.89±0.36	9.31±0.18	9.15±0.19	9.32±0.20	9.17^{x}±0.12
	Mid Cycle $PGF_2\alpha$ inj.	C	4	9.47±0.24	--	9.42±0.23	9.55±0.10	9.48±0.11
		NC	4	8.59±0.20	--	9.25±0.25	9.10±0.25	8.98±0.15
		Pooled	8	9.03±0.22	--	9.33±0.16	9.33±0.15	9.23^{x}±0.10

	Normal Cyclic Control	C	4	--	--	9.70±0.22	9.60±0.24	9.65±0.15
		NC	4	--	--	9.84±0.09	9.72±0.18	9.78±0.96
		Pooled	8	--	--	9.77±0.11	9.66±0.14	9.71^{y}±0.09
Buffalo	Ovsynch	C	4	9.53±0.52	9.80±0.30	10.12±0.21	10.10±0.10	9.89±0.16
		NC	4	9.88±0.21	10.05±0.23	9.65±0.40	9.93±0.18	9.87±0.13
		Pooled	8	9.70±0.27	9.93±0.18	9.88±0.23	10.01±0.10	9.88^{y}±0.10
	Mid Cycle $PGF_2\alpha$ inj.	C	5	9.30±0.13	--	9.32±0.36	9.81±0.14	9.48±0.14
		NC	3	9.01±0.00	--	8.98±0.04	9.22±0.10	9.07±0.05
		Pooled	8	9.19±0.09	--	9.20±0.23	9.59±0.14	9.33^{x}±0.10
	Normal Cyclic Control	C	4	--	--	9.04±0.23	9.10±0.23	9.07±0.15
		NC	4	--	--	9.70±0.12	9.66±0.14	9.68±0.09
		Pooled	8	--	--	9.37±0.17	9.38±0.16	9.37^{x}±0.12

C = conceived, NC = non-conceived; Day-0 = Day of treatment, D-AI = Day of AI, D-21 = Day 21 post-AI; Means bearing uncommon superscripts within the row (a,b) and column (p,q) (x,y) differ significantly (P<0.05).

Table 4: *Mean Plasma Inorganic Phosphorus Levels (Mg/Dl) in Repeat Breeding Cows and Buffaloes under Ovsynch Protocol and Mid-Cycle $PGF_2\alpha$ Inj. on Different Days of Treatment/AI*

	Treatment Protocol	Status	No	Days from Treatment/AI				Overall
				D-0	D-7	D-AI	D-21	
Cow	Ovsynch	C	5	4.40±0.35	4.61±0.38	4.20±0.30	4.65±0.13	4.47±0.15
		NC	3	4.53±0.12	4.77±0.21	4.05±0.04	4.23±0.25	4.39±0.11
		Pooled	8	4.45±0.22	4.67±0.24	4.14±0.18	4.49±0.14	4.44^{x}±0.10
	Mid Cycle $PGF_2\alpha$ inj.	C	4	4.74±0.12	--	4.71±0.11	4.77±0.05	4.74±0.05
		NC	4	4.29±0.10	--	4.62±0.13	4.55±0.13	4.49±0.08
		Pooled	8	4.51±0.11	--	4.67±0.08	4.66±0.08	4.61^{xy}±0.05
	Normal Cyclic Control	C	4	--	--	4.88±0.10	4.72±0.17	4.80±0.09
		NC	4	--	--	4.93±0.06	4.99±0.03	4.96±0.03
		Pooled	8	--	--	4.90±0.05	4.85±0.09	4.88^{z}±0.05
Buffalo	Ovsynch	C	4	4.76±0.26	4.90±0.15	5.06±0.10	5.04±0.05	4.94±0.08
		NC	4	4.94±0.11	5.03±0.12	4.82±0.20	4.97±0.09	4.94±0.06
		Pooled	8	4.85±0.13	4.96±0.09	4.94±0.11	5.01±0.05	4.94^{y}±0.05
	Mid Cycle $PGF_2\alpha$ inj.	C	5	4.65±0.06	--	4.66±0.18	4.91±0.07	4.74±0.07
		NC	3	4.51±0.00	--	4.57±0.10	4.61±0.05	4.56±0.03
		Pooled	8	4.60±0.05	--	4.63±0.11	4.79±0.07	4.67^{x}±0.05
	Normal Cyclic Control	C	4	--	--	4.53±0.23	4.76±0.13	4.64±0.13
		NC	4	--	--	4.94±0.05	4.85±0.07	4.90±0.04
		Pooled	8	--	--	4.73±0.13	4.80±0.07	4.77^{x}±0.07

C = conceived, NC = non-conceived; Day-0 = Day of treatment, D-AI = Day of AI, D-21 = Day 21 post-AI; Means bearing uncommon superscripts within the row (a,b) and column (p,q) (x,y) differ significantly (P<0.05).

Ahlawat (2003) reported the mean calcium levels in conceiving and non-conceiving crossbred cows to be 9.09±0.25 and 8.59±0.38 mg/dl. These findings were similar to the present findings in both repeat breeding cows and buffaloes. The findings of Chandrakar et al. (2003) in repeat breeder cows were also in close corroboration with the present findings. Parmar (2013) reported higher mean plasma calcium for postpartum buffaloes under $PGF_2\alpha$ treatment than the control group (9.98±0.04 vs 9.17±0.04 mg/dl; P<0.05). Similar were the findings of Savalia et al. (2013) in repeat breeding buffaloes under Mid-cycle $PGF_2\alpha$ treatment. Patel et al. (2014[b]) reported mean plasma calcium levels of 9.58±0.17 and 9.90±0.12 mg/dl in conceived and non-conceived repeat breeder cows, which also closely corroborated with the present findings. Parmar (2013) reported the mean plasma calcium levels to be 8.66 ± 0.15 mg/dl and 8.57 ± 0.15mg/dl in non-conceived and conceived repeat breeding buffaloes under Mid-cycle $PGF_2\alpha$ inj. group.

Fayez et al. (1992) reported that repeat breeder Egyptian buffaloes had significantly lower inorganic phosphorus concentration than normal animals. Parmar (2013) obtained much higher mean inorganic

phosphorus levels as 6.15±0.28 mg/dl and 7.25±0.50 mg/dl in non-conceived and conceived repeat breeding buffaloes under Mid-cycle $PGF_2\alpha$ injection group than the present findings. Patel et al. (2014[b]) could not find significant variation in inorganic phosphorus concentration between various sampling days or between conceived and non-conceived groups of crossbred cows under Mid-cycle $PGF_2\alpha$ treatment, which is in line with the present findings.

From the results, it can be inferred that the application of both Ovsynch protocol and Mid-cycle $PGF_2\alpha$ injection are good tools for induction of timed estrus and ovulation and enhancement of pregnancy rate in repeat breeding cows and buffaloes without significantly influencing the plasma biochemical and mineral profile. However, looking to the results, cost and time involved, Mid-cycle $PGF_2\alpha$ treatment is much economic and cost effective as compared to Ovsynch protocol in handling problem breeders under field conditions.

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Research Article | Open Access

Histology of Sinoatrial Node in the Dromedary Camel Foetus

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Abstract The histological structure of the sinoatrial node (SAN) in the dromedary camel foetus was investigated using routine histological techniques and some special stains. Twenty foetuses were used. They were divided into two groups 10 foetuses were in the second trimester (131-260 days); the rest in the third trimester (261-423.5 days of gestation). Samples were collected from the right atrium cranial to the opening of cranial vena cava. The SAN in the camel foetus was found in subepicardial region cranial to the opening of cranial vena cava at the junction between the cranial vena cava and right atrium. Two types of cells were observed; the first type had dark cytoplasm and large spherical lightly stained nucleus. The cells of the second type were small and spindle in shape with dark small nuclei. It is concluded that SAN in camel foetuses in the second and third trimesters had the same location as in the adult and also had two types of cells as in other animal species.
Keywords *Camel; Foetus; Histology; Sinoatrial Node*

1. Introduction

It is well known that SAN in the adult animals is located in the upper part of the right atrium at the junction between the right atrium and the opening of the cranial vena cava; its function is to provide the electrical impulse responsible for normal cardiac rhythm (Ghazi and Tadjalli, 1996; Sánchez-Quintana and Yen Ho, 2003; Nabipour, 2012).

Walls (1947) studied the development of the specialized conducting tissue of the human heart and found that the sinus node can be identified at 10 mm foetus. The sinus node of yak was studied using the histological methods (Duan et al*.,* 2012).

Histological features of the sinus node include the cells, nodal artery, collagen framework, and nerves (Nabipour, 2012).

In the heart of the adult dromedary camel the sinus node was located 0.5 mm beneath the epicardium, near the junction between the cranial vena cava and the right atrium at the sulcus

terminalis (Ghazi and Tadjalli, 1996). Histologically, the authors noted that the sinus node of the camel contained a central artery and a framework of collagen fibres which were distributed around the central artery.

To our knowledge no studies were found concerning the camel foetus. Hence, this study was undertaken. The objective of this research was to investigate the histological structure of SAN of the dromedary camel foetus.

2. Materials and Methods

Twenty hearts of camel foetuses obtained from Tamboul slaughterhouse, Gezeira, Sudan were used in this study. Depending on the age, the foetuses were divided into two groups: second trimester (131-260 days) and third trimester (261-423.5 days). The age of the foetus was determined using the equation of crown vertebral-rump length (CVRL) as described by Elwishy et al. (1981).

Small pieces (about $1cm^3$ thick) were taken from the right atrium cranial to the opening of cranial vena cava at the junction between the cranial vena cava and right atrium. Specimens were fixed in 10% buffered formalin. They were then processed by routine histological procedures and stained with H and E. Some special stains including Van Geison's for collagenous fibres and Verhoeff's for elastic fibres were applied (Bancroft and Stevens, 2008).

3. Results and Discussion

According to the available literature the present study reports for the first time that the SAN is present at the opening of cranial vena cava during the second and third trimesters of the dromedary camel foetus. It appears that the location of the SAN did not change during development in camels since its position in the foetus (this study) and adult hearts (Ghazi and Tadjalli, 1996; Ghonimi et al., 2015). It also remained the same in human (Titus, 1973), that is to say in the subepicardial region at the opening of the cranial vena cava in the right atrium. However, Walls (1947) stated that SAN can be found in upper part of the bases of both vena cavae in human foetuses.

SAN has been studied in many animals by different histological techniques which revealed a general agreement about its location, shape and cellular structure (Ghazi and Tadjalli, 1996; Nabipour, 2012; Ghonimi et al., 2015).

In the present investigation in the second trimester 131- 260 days SAN appeared as a group of cells connected to each other with a connective tissue (Figures 1 and 2). They had a dark cytoplasm and peripheral nuclei. There were no intercalated discs observed. At the stage of 169 days (Figure 1) and 251 days of gestation (Figure 2) SAN was found beside the ordinary cardiac muscles and embedded in a connective tissue. SAN artery was found beside SAN also (Figure 1). Two cell types were identified: the first type had dark cytoplasm and peripheral nuclei, the second type consiseted of small cells with dark and small central nuclei (Figures 1 and 2).

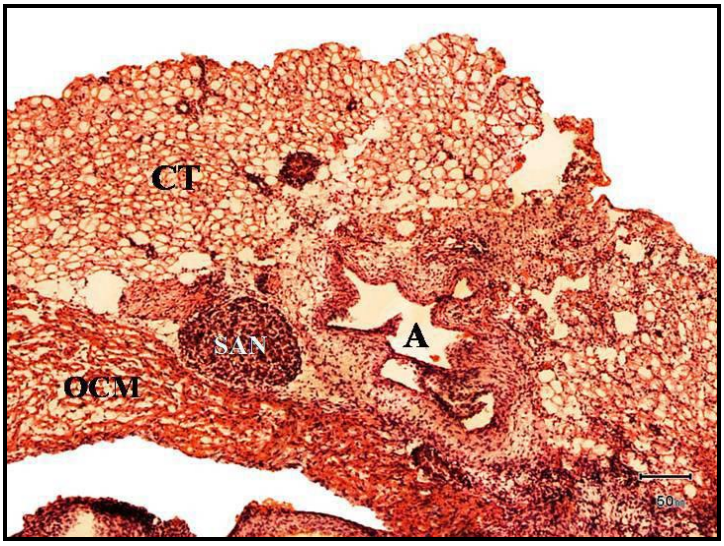

Figure 1: *Photomicrograph of Right Atrium of Camel Foetus 38 Cm CVRL (169 Days of Gestation) Showing a SAN Artery, Connective Tissue (CT) Surrounding SAN and Ordinary Cardiac Muscle (OCM). H and E. X40*

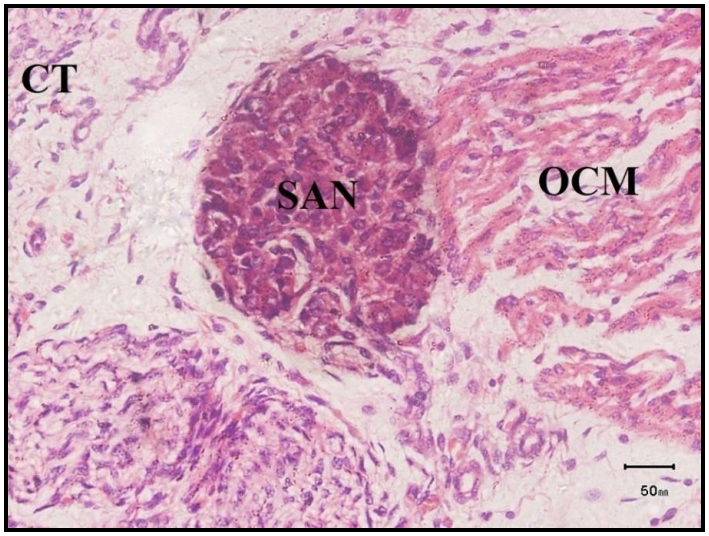

Figure 2: *Photomicrograph of Right Atrium of Camel Foetus 68 Cm CVRL (251 Days of Gestation) Showing Connective Tissue (CT) Surrounding SAN and Ordinary Cardiac Muscle (OCM). H and E. X40*

In the early stages of the third trimester (261- 423.5 days), SAN appeared as a group of dark stained fibres (Figure 3). It was embedded in fair amounts of connective tissue that contained adipose cells and collagenous fibres. At stages of 268 days and 273 days of gestation the cells were surrounded by a circular and very light area and they had different shapes and sizes (Figures 3 and 4). Their nuclei were large and were peripherally located. The nuclear chromatin was peripherally concentrated (Figures 3 and 4). The connective tissue surrounding SAN was rich in blood capillaries and collagenous fibres. An arteriole and a venule were embedded between ordinary cardiac muscles and SAN connective tissue (Figure 3). Similarly, Ghazi et al. (1998) found that the sinus node of cats contained normally dense collagen framework. However, Ghonimi, et al. (2015) claimed that the SAN of camel consisted of stroma and parenchyma. James (1970) stated that the sinus node was originated in the sinus venosus but subsequently a dense collagen framework developed. He added that partitioning of the sinus node by collagen was important not only to the nature of its cellular development but also to its pacemaking function. This is in confirmation with our findings in camel foetus.

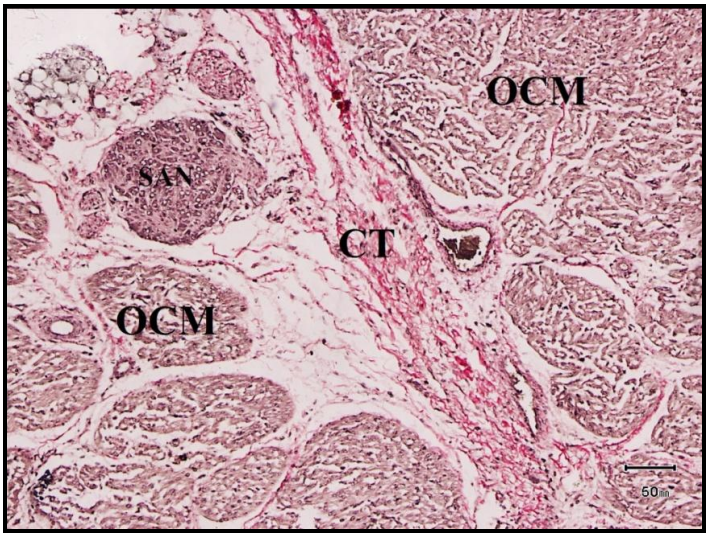

Figure 3: *Photomicrograph of Right Atrium of Camel Foetus; 74 Cm CVRL (268 Days of Gestation) Showing SAN With Framework of Connective Tissue (CT) Rich With Collagen Fibres (CT) and Blood Vessels Embedding Between SAN and Ordinary Cardiac Muscle (OCM). CT: Connective Tissue. Verhoeff's stain (X10).*

In the late stages of the third trimester (261- 423.5 days) SAN became elongated in shape with long peripheral tapering ends (Figures 4 and 5). Similarly, in equine, Bishop and Cole (1967) found that SAN had a body and long acuminate cranial and caudal crura. However, in the domestic cat it was described as triangular in shape (Ghazi et al., 1998).

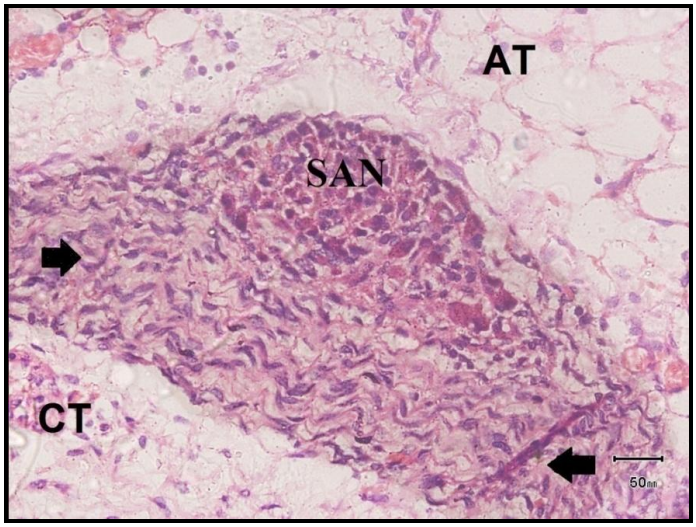

Figure 4: *Photomicrograph of Right Atrium of Camel Foetus; 76 Cm CVRL (273 Days of Gestation) Showing SAN with Elongated Ends (Arrows) Embedding in Connective Tissue (CT) With Adipose Tissue (AT). H and E. (X40)*

Two types of cells can be identified; the first type had dark cytoplasm and large spherical light nuclei with peripherally located chromatin (Figure 5, a and b). The other cells were small and spindle in shape with dark small nuclei. Blood capillaries were found in between these cells (Figure 5, a and b). This is in consistency with Ghazi et al. (1998).

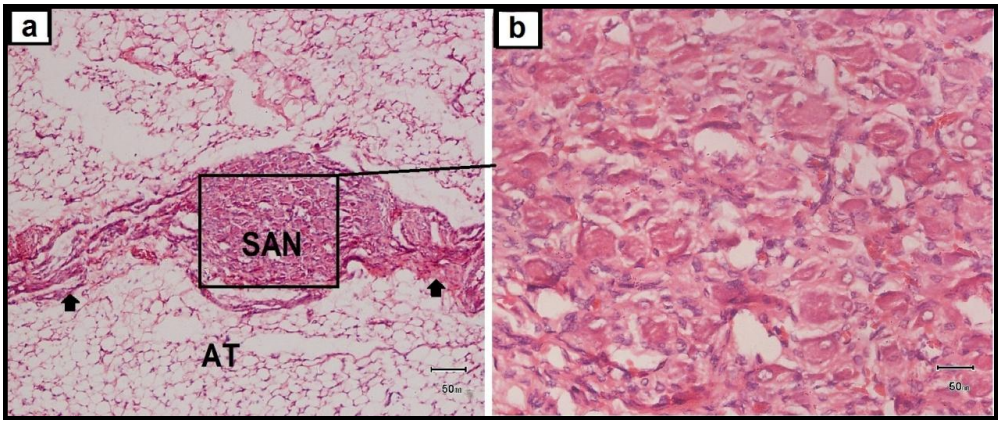

Figure 5: *Photomicrographs of Right Atrium of Camel Foetus; 131 Cm CVRL (423 Days of Gestation) Showing SAN with Tapering Ends (Arrows) Surrounding by Adipose Tissue (AT) Van Gieson's stain. X10*

A ganglion associated with SAN in the third trimester specimens at (273 – 283 days of gestation) appeared in the subepicardial area near the cardiac muscles embedded in connective tissue rich in adipose tissue, blood vessels and nerves (Figure 6 a and b). The ganglion was surrounded by a thin connective tissue capsule that encloses large scattered irregular neurons characterized by large nucleus and prominent nucleolus. Satellite cells and axons were observed between the neurons (Figure 6, b). According to Nabipour (2012) human, horse, goat, dog and domestic cat SAN ganglia were present at the periphery of the node. Earlier, Nonidez (1943) stated that SAN was supplied by axons of neurons of the intrinsic cardiac ganglia.

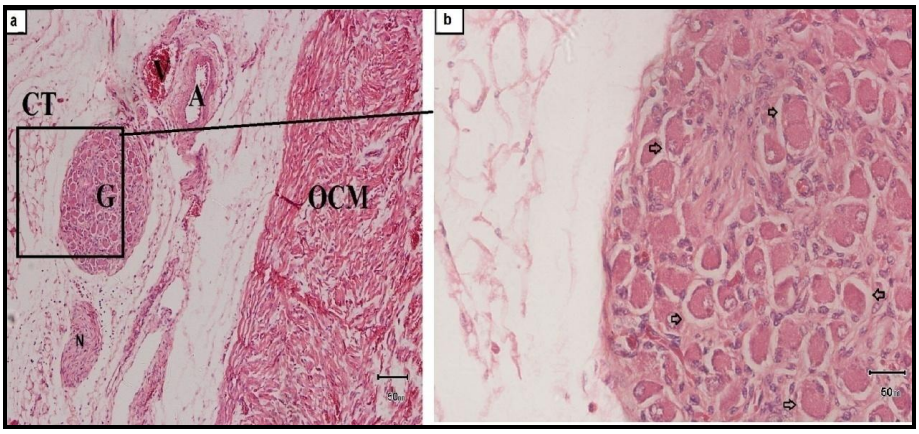

Figure 6: *Photomicrographs of Capsulated Ganglion of the SAN in the Heart of Camel Foetuses in 79.5 Cm CVRL (283 Days of Gestation). Note the Large Nucleus (Arrows) Perikaryon and Blood Vessels (A, V) and Nerves (N) in the Surrounding Connective Tissue Van Gieson's stain. X10*

4. Conclusion

It is concluded that SAN in camel foetuses in the second and third trimesters had the same location as in the adult and also had two types of cells (P) and (T) as in other animal species. SAN ganglion was first observed in the third trimester of gestation.

Acknowledgements

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Research Article **Open Access**

Molecular Characterization of *Corynebacterium bovis* causing Clinical Mastitis and Increasing Somatic-Cell Count

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Abstract Bovine mastitis remains the most economically important disease affecting dairy cows. Bacteria of the genus *Corynebacterium* spp., especially *Corynebacterium bovis* (*C. bovis*), are frequently isolated from bovine mastitis although there are divergent reports about the role of this pathogen in bovine mastitis. In the present study we report the presence of new *C. bovis* strains from milk exhibiting increased SCC of 12 cows with subclinical and clinical mastitis. Irregular Gram-positive bacilli isolated in pure cultures underwent conventional biochemical tests which generated indefinite phenotypic profiles (*Corynebacterium mastitidis/C. bovis*). API-Coryne System 3.0 identified all the milk isolates as *C. bovis* (code number 4501014; 99.9% confidence level). Phylogenetic analysis based on *16S rRNA* and *rpo*B sequences confirmed the identification of the strains as *C. bovis*.
Keywords *Corynebacterium bovis; Mastitis; Sequencing of Genes; 16S rRNA; rpoB*

1. Introduction

Bovine mastitis remains the most economically important disease affecting dairy cows. Coryneform bacteria are frequently isolated from bovine mastitis, and lipophilic *Corynebacterium bovis* has been the most frequently isolated microorganism of this group (Watts et al., 2000, 2001). *C. bovis* isolation has been positively correlated to the increase of SCC in milk samples from cows with subclinical mastitis (Brooks and Barnum, 1984; Pankey et al., 1985; Sordillo et al., 1989). The economic loss varies with the degree of alveolar epithelial lesion and the increase in leukocyte infiltration, which decreases milk secretion (Hallberg et al., 1995).

Since there are still some conflicting research results concerning the relevance of *C. bovis* as the etiological agent of mastitis, in the present study we report different cases of clinical and subclinical mastitis due to *C. bovis* during an outbreak in a dairy farm. Phylogenetic analysis, based on 16S rRNA sequences, unambiguously demonstrated that the clinical isolates of different phenotypes belonged to *C. bovis* species.

2. Materials and Methods

2.1. Milk Collection

During a mastitis outbreak in a dairy farm located in the central region of São Paulo State, Brazil, screening tests were carried out to detect clinical and subclinical mastitis (positive strip cup test = clinical mastitis; California Mastitis Test-CMT score higher than one cross = subclinical mastitis) among 300 Holstein animals, of different ages and at different lactation stages. Subsequently, 106 milk samples were aseptically collected from mammary quarters showing CMT score +++ and positive strip cup test for microbiological tests and SCC.

2.2. Microbiological Culture Conditions

Milk samples (0.1 mL) were sown in agar base (Difco™) added of 5% ovine blood and incubated at 37ºC for 24-72h in aerobiosis. *Corynebacterium*-like colonies were initially identified according to Gram staining, colonial morphology, pigmentation and hemolytic properties. Colonies of irregular Gram-positive rods (IGPR) were re-cultured in brain heart infusion (BHI) added of 1% tween-80 and incubated for 24h. Then, the microorganisms were subjected to phenotypic and genotypic tests for identification as described below.

2.3. Phenotypic Tests for Corynebacteria Identification

Conventional biochemical characterization was done by means of tests of lipophilia, catalysis, O/F metabolism, acid production from glucose, maltose, sucrose, trehalose, ribose, mannitol, lactose, xylose, mannose, arabinose, fructose, galactose and glycogen; hydrolysis tests of esculin, urea, tyrosine, in addition to assays of alkaline phosphatase, pyrazinamidase, nitrate reduction and CAMP reaction using *Staphylococcus aureus* sample (Murray, 2010). ONPG (o-Nitrophenyl-ß-*D* galactopyranoside) hydrolysis test was carried out with the enzyme ß-galactosidase produced by *C. bovis*, considered decisive for its differentiation according to Holt et al. (1994).

Microorganisms were also characterized according to the commercially available semi-automated identification API-Coryne System 3.0 (bioMerieux, Lyon, France) by following the manufacturer's instructions; decoded by the API web system (https://apiweb.biomerieux.com)

2.4. Gene Amplification and Sequencing

C. bovis identification was reconfirmed by sequencing *16S rRNA* and *rpo*B genes. Each strain was grown in BHI broth under incubation for 24-48h at 30°C and centrifugation for 5 min at 3000 rpm. The pelleted bacteria was suspended in 500 µL sterile water and subsequently boiled during 15 min for DNA extraction. Then, cell extracts were immediately stored at -20ºC until used in PCR reactions. *16S rRNA* gene was amplified by using the universal primers: pA (5'-AGA GTT TGA TCC TGG CTC AG) and pH (5'-AAG GAG GTG ATC CAG CCG CA), as described by Watts and co-workers (2000). The PCR product was purified in both directions by primer walking with the oligonucleotides using the following primers for sequencing: 1831 (5'- GAG GAA CAC CGA TGG CGA AGG C), 1832 (5'- GCC CCC GTC AAT TCC TTT GAG TT) (Watts et al., 2000), 519r (5'- G(AT)A TTA CCG CGG C(GT)G CTG), and 1242f (5'- CAC ACG TGC TAC AAT GG) (Johnson, 1994). *rpo*B gene was amplified and

sequenced with primers as previously described by Khamis et al., 2004. Sequencing reactions were performed with BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) on an ABI-3730 Automated DNA Sequencer (Applied Biosystems), according to standard protocols. 16S rRNA gene sequences were compared to those available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm and the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/html). *rpo*B gene sequences were only compared in GenBank database.

2.5. Phylogenetic Analysis

16S rRNA gene sequences were aligned by CLUSTALX (Thompson et al., 1997). The phylogenetic trees were prepared by using the neighbor-joining genetic distance method and the software MEGA 4.0 package with the option of complete deletion of gaps (Tamura et al., 2007). Kimura two-parameter model was chosen for all NJ tree constructions. The reliability of each tree topology was checked by 1000 bootstrap replications.

3. Results

Results related to CMT, SCC/mL milk and mastitis classification are shown in Table 1. Twelve IGPR strains were isolated from different animals, three of which had signs and symptoms of clinical mastitis and nine had subclinical infections. In all opportunities, microorganisms were isolated from milk in pure culture and at concentrations higher than 30 CFU/0.1mL, showing small whitish non-hemolytic colonies of 0.2 to 0.5 mm diameter after 72-h incubation with dried aspect. Bacterioscopy according to Gram's method indicated Gram-positive cocobacilli forms.

Table 1: *California Mastitis Test-CMT and somatic cell count-SCC milk results and type of mastitis of 12 cows during an outbreak in a farm located in Botucatu, São Paulo, Brazil, 2012*

Record	Animal/teat	Mastitis	CMT (score)	SCC (x1000/mL)
42/11 – 39	1462/PR	Clinical	ND	ND
42/11 – 65	1664/PR	Clinical	ND	ND
42/11 – 66	139/PR	Clinical	ND	ND
43/11 – 13	1589/PL	Subclinical	3	1567
43/11 – 32	1385/PR	Subclinical	3	4765
43/11 – 88	1600/PR	Subclinical	2	4908
43/11 – 90	1367/AL	Subclinical	3	7815
43/11 – 91	1445/PL	Subclinical	2	498
43/11 – 98	1463/PR	Subclinical	3	856
43/11 – 99	1106/PL	Subclinical	3	8631
43/11 – 100	1520/PL	Subclinical	3	6389
43/11 – 107	1591/PR	Subclinical	3	478

ND = Not done; SCC = somatic-cell count mL/milk; AR = anterior right teat; AL = anterior left teat; PR = posterior right teat; PL = posterior left teat

All 12 IGPR samples showed lipophilic properties and had negative results for esculin hydrolysis test, nitrate reduction and CAMP; there was no acid production in the presence of the carbohydrates glucose, maltose, sucrose, mannitol, trehalose, xylose, arabinose, ribose, fructose and glycogen, besides weak urea hydrolysis. These results were compatible with the species *Corynebacterium mastitidis* (Holt et al., 1994; Fernandez-Garayzabal et al., 2001; Murray, 2007). On the other hand, the microorganisms showed the following results suggestive of *C. bovis:* growth in the presence of 6.5% NaCl, positive ONPG (β-galactosidase) and negative pyrazinamidase activity. Further analysis by the API-Coryne system identified all 12 strains as *C. bovis* by the code number 4501014 with 99.9% confidence level.

Results of the sequencing of genes *16S rRNA* and *rpoB* from the isolates (Table 2) indicated that the samples had similarity superior to 98.7% for gene 16S rRNA only with the species *C. bovis*

(Stackebrandt & Ebers, 2006). Phylogenetic analysis unambiguously demonstrated that the clinical isolates belonged to *C. bovis* species, as illustrated in Figure 1. The similarity values obtained in the analysis of gene *rpo*B also confirmed the data obtained in the analysis of gene 16S rRNA. The gene sequences of *16S rRNA* and *rpoB* were deposited in GenBank under the numbers JX298782 and JX298783 for sample 42/11 - 65; JX298784 and JX298785 for sample 42/11 - 66; JX298786 and JX298787 for sample 43/11 - 99; JX298788 and JX298789 for sample 43/11 - 100.

***Table 2**: Sequence analysis based on Corynebacterium spp. sequences of 16S rRNA and rpoB genes*

Strain number	bp	16S rRNA similarity (%)	bp	rpoB similarity (%)
42/11 - 65BR	1507	100.00 *Corynebacterium bovis*	421	98.57 *Corynebacterium bovis*
42/11 - 66BR	1500	100.00 *Corynebacterium bovis*	388	98.45 *Corynebacterium bovis*
43/11 - 99BR	1506	100.00 *Corynebacterium bovis*	393	98.47 *Corynebacterium bovis*
43/11 - 100BR	1506	99.93 *Corynebacterium bovis*	400	98.50 *Corynebacterium bovis*

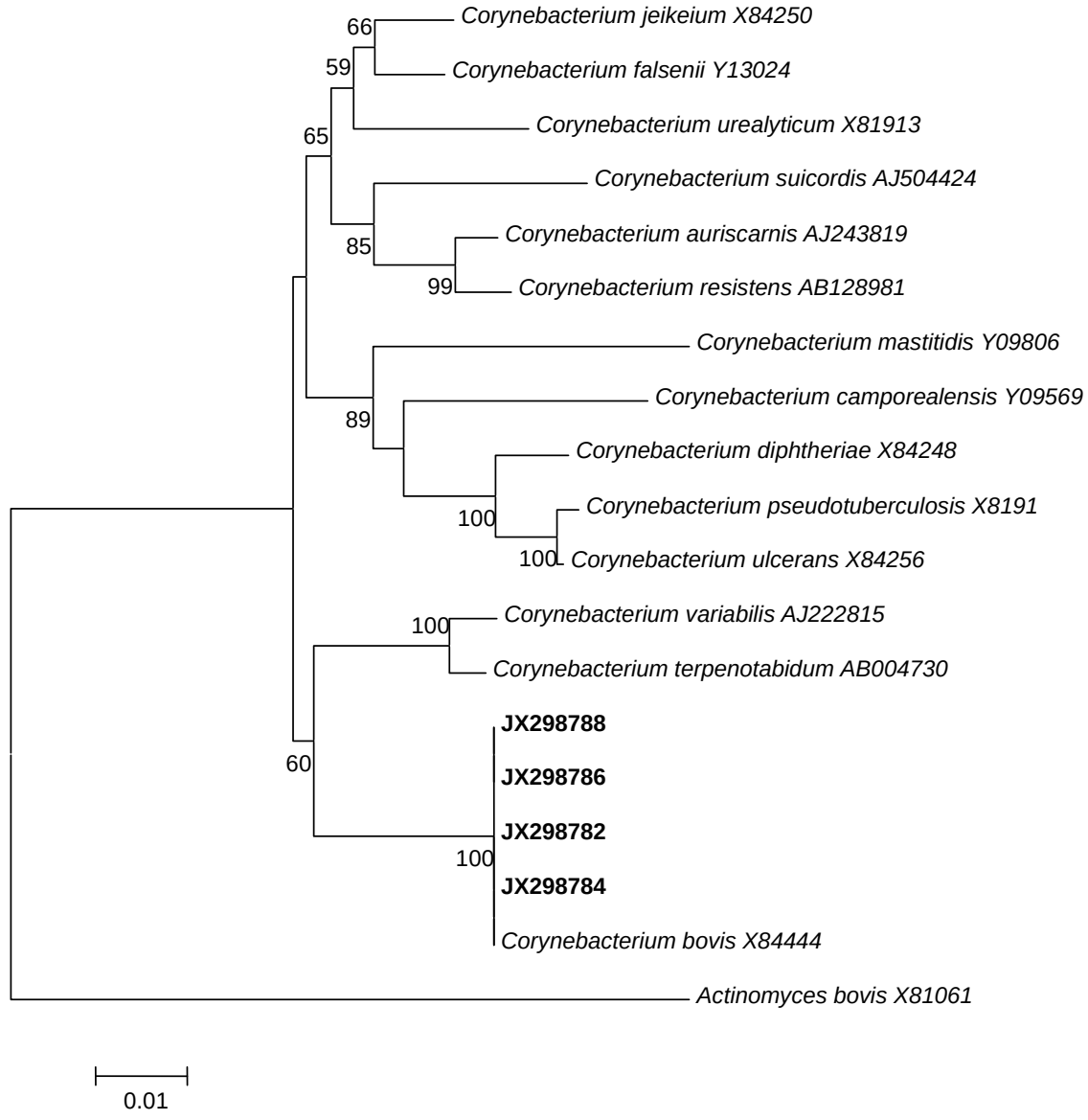

***Figure 1**: Phylogenetic tree based on the neighbor-joining method using 16S rRNA gene sequences. Distances were estimated by using the Kimura two-parameter model. Bootstrap percentages after 1,000 simulations are shown. The Actinomyces bovis (T) X81061 sequence was used as outgroup*

4. Discussion

Based on the assessment of the dynamics of intramammary infection by *C. bovis*, Honkanen-Buzalski et al. (1984) assumed that there is no protection against major pathogens and concluded that *C. bovis* is relevant in the pathogenesis of bovine mastitis. Some authors highlight its effects regarding decreased milk production and increased SCC/mL milk (Costa et al., 1986; Viseslava and Vera, 1989; Hallberg et al., 1995; Wilson, 2001). Mettifogo et al. (1991) evidenced the importance of *C. bovis* in subclinical mastitis by isolating it from 25.77% examined milk samples with mean microscopic SCC of 1,032,429 cells/mL milk. In the same region, Beloti et al. (1997) found *C. bovis*, among other pathogens, in 18.98% subclinical cases of mastitis, with mean SCC of 979 x 10^3 cells/mL milk, showing cell response in intramammary infection.

However, *C. bovis* is considered, by some authors, a pathogen of less importance in the etiology of bovine mastitis, or even a commensal of the udder, located especially in the teat duct (Pankey et al., 1985; Bexiga et al., 2011) and capable of protecting it during the invasion of other microorganisms (Bramley et al., 1976; Huxley, 2003) such as *Staphylococcus aureus*, which is considered a major pathogen.

The occurrence as outbreaks or not shows the relationship between *C. bovis* infectivity and pathogenicity characteristics. In addition to these aspects, there is decreased production, as demonstrated by the comparative analysis of the production of teats infected with *C. bovis* with that of their negative homologues (Domingues et al., 1998). Data showed 23.5% reduction in the production of infected teats, which evidenced the risk represented by this agent in the development of dairy farming.

Reasserting the participation of this microorganism in mammary pathology, Costa et al. (1986) recovered it from 32.06% and 22.74% cases of clinical mastitis and subclinical mastitis, respectively, and Langoni et al. (1998) from 12.08% and 15.94% of the 850 studied cases of clinical mastitis and 7902 cases of subclinical mastitis, respectively.

Another aspect that reinforces its pathogenicity injuring the mammary alveolar tissue is the decrease in milk production, as previously reported (Langenegger et al., 1981; Domingues et al., 1998). Association of *C. bovis* with bovine mastitis was also reported by Ferreiro et al. (1981), and Costa et al. (1985) argued that, besides the high prevalence (32.5%) indicating its infectivity, the agent was isolated in pure culture of cases of clinical mastitis (28.18%) and subclinical mastitis (27.5%).

The present results reinforce the potential of *C. bovis* as mastitis agent also due to its pure and exuberant isolation in both cases of clinical mastitis and cases of subclinical mastitis with high SCC, in some cases reaching values superior to 1000 x 10^3SCC/ml milk, which agrees with Mettifogo et al. (1991), Beloti et al. (1997), Harmon (1998) and Wilson (2001).

Molecular diagnosis has brought great advances, constituting a fundamental tool for molecular epidemiology studies but requiring the use of reagents and specific primers, besides previous standardization, appropriate laboratories and professional experience (Bexiga et al., 2011). The present results indicated the use of the API-Coryne System as an alternative tool for the identification of *C. bovis* in cases of bovine mastitis outbreaks.

5. Conclusion

In conclusion, the differentiation and the diagnosis of *C. bovis* must be grounded in well conducted studies that meet the assessment criteria related to milk production by the affected quarters, as well as in SCC/mL milk, and fundamentally characterization must be appropriate by means of genotypic

studies and genetic sequencing for adequate interpretation of results, leading to more pertinent conclusions as to its role in bovine intramammary pathogenicity.

Acknowledgements

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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Pulsed Wave Doppler and Color Flow Doppler Evaluation in Healthy Dogs and Dogs with Cardiac Disease

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Abstract Doppler echocardiography gives a physiological assessment of blood flow within the cardiac chambers, across valve orifices and in the great vessels. Pulsed wave Doppler can detect flow pattern in a very discrete region of simultaneously imaged 2-D echocardiographic plane. Twenty apparently healthy dogs and twenty affected dogs with dilated cardiomyopathy and mitral valve insufficiency ten each were selected for the study and subjected to detailed color flow and pulse wave Doppler study. In color flow Doppler studies a normal mitral and tricuspid flow were red in color as the flow is towards the transducer and when the nyquist limit exceeded it had a layers of blue superimposed on it. Aortic flow was seen as hues of blue and red and because of depth of the aorta nyquist limit was low and aliasing was commonly seen. In pulmonary flow the flow was usually blue in color as it was away from the transducer and total reversal of color was often seen as flow progress from high velocity during early systole to the lower velocities at the end of systole. In mitral valvular insufficiency two out often dogs showed jet occupying less than 20 per cent of atrium, three out of ten dogs showed jet occupying 20-50 per cent of atrium and five out of ten dogs showed jets occurring more than 50 per cent of atrium. In dilated cardiomyopathy mild to moderate jets were appreciated in four out of ten dogs in both mitral and tricuspid valves and two out of ten dogs showed mitral regurgitation alone.

Keywords *Colour Flow Doppler; Pulse Wave Doppler; Dogs*

1. Introduction

Colour flow Doppler imaging used pulsed wave technology to build a colour coded image of blood flow velocity superimposed on 2-D or M-mode anatomic image of the heart (Kirberger et al., 1992) Colour flow Doppler offers several advantages over the pulsed wave Doppler. First, region of normal and abnormal flow can be identified much faster because it cover a much greater area in each sample, thus increasing the efficacy of examination. Regurgitation jets and shunts are more rapidly identified and localized. Second, the colour display may be used to determine the direction of jet and help to align pulsed wave Doppler beam to the jet for more accurate velocity measurement. Finally colour flow display is more anatomically familiar and comprehensible to inexperienced examiners. To familiarize this technique among practicing veterinarians and to assess its viability in diagnosing heart

diseases in dogs. The present study was designed with following objectives: To evaluate the blood flow velocities and colour flow pattern at the level of the mitral, tricuspid, aortic and pulmonary valve in normal dogs and to document pulsed wave doppler velocities and colour flow in dogs with Dilated cardiomyopathy and Mitral valvular insufficiency.

2. Materials and Methods

Twenty apparently healthy adult dogs of different breeds, sexes and age groups attending the outpatient unit for general health checkup and vaccination were selected for the study. The breeds selected were Spitz (seven dogs), Labrador retriever (7 dogs) and non-descript (six dogs). The dogs in this group were evaluated for normality by physical examination; lead II electrocardiograph, clinical laboratory values and two dimensional echocardiography. Apart from the normal study, ten dogs with dilated cardiomyopathy and ten dogs with mitral valvular insufficiency were selected for the study. Both healthy and diseased dogs were subjected to thorough pulsed wave Doppler and colour flow Doppler studies. The ultrasound machine used for the study was ALOKA Prosound SSD-3500SV with micro convex probe of frequency ranging from 3 to 6 MHz Along with the 2D imaging it has facilities of pulse wave Doppler and color flow Doppler.

2.1. Procedure for Doppler Examination

Three Doppler windows were used to get as parallel to blood flow as possible. These were the left caudal or apical four chamber view, the left caudal or apical five chamber view and the right parasternal short axis view at aortic valve level. Dogs were clipped over the examination area and placed on a specially constructed table for echocardiographic examination enabling all examination to take place from below the patient. All measurements were done by pulsed wave Doppler and colour flow Doppler.

The ultrasound machine used for the study was ALOKA Prosound SSD-3500SV with micro convex probe of frequency ranging from 3 to 6 MHz. The dogs were conscious throughout the examination and no drugs were used. The smallest cursor sampling seize (2 mm) was used to minimize artifact from the movement of cardiac structure (Kostucki et al., 1986). All the measurements in pulsed wave Doppler were taken in three sequential individual beats during normal sinus rhythm and averaged. Measurements were performed using electronic calipers on frozen screen images. Colour flow Doppler was superimposed on the same two dimensional images obtained for pulsed wave Doppler measurement. Imaging Plane and Sample Volume Position for Different Flow Areas (Kirberger et al., 1992) (Figure 1)

Mitral Valve Flow

Imaging plane: Left caudal (apical) four chamber view. Sample volume position: In the left ventricle just distal to the mitral annulus at the point of maximal opening of the mitral valve (Figure 1).

Left Atrial Flow

Imaging plane: Left caudal (apical) four chamber view. Sample volume position: One quarter of distance between the mitral annulus and the dorsal wall of the left atrium (Figure 1).

Tricuspid Valve Flow

Imaging plane: Left caudal (apical) four chamber view. Sample volume position: In the right ventricle just distal to the tricuspid annulus at the point of maximal opening of tricuspid valve (Figure 1).

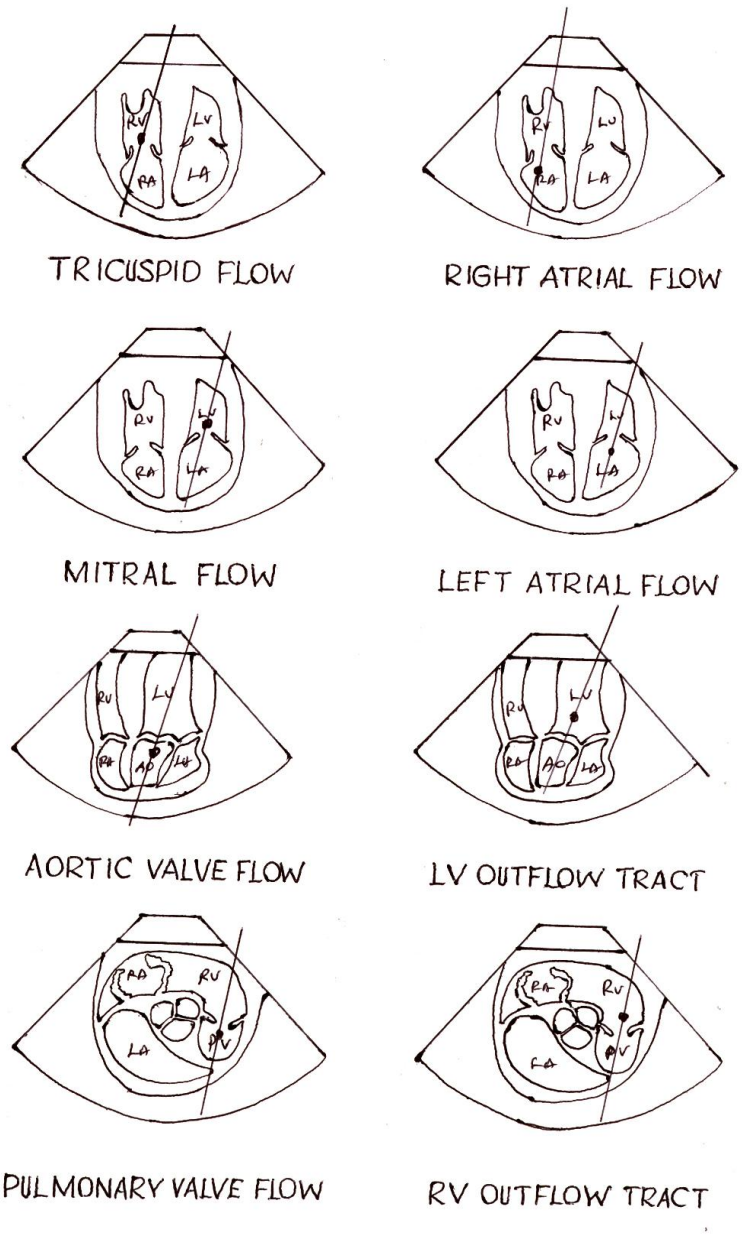

Figure 1: *Normal sample volume positions for Doppler studies*

Right Atrial Flow

Imaging plane: Left caudal (apical) four chamber view. Sample volume position: One quarter of the distance between the tricuspid annulus and the dorsal wall of right atrium (Figure 1).

Aortic Valve Flow

Imaging plane: Left caudal (apical) long axis view of left ventricular outflow region. Occasionally an apical five chamber view was used to align more parallel to the ascending aorta. Sample volume position: In the ascending aorta at the distal end of the sinus of Valsalva (Figure 1).

Left Ventricular Outflow Tract Flow

Imaging plane: Left caudal (apical) long axis view of left ventricular outflow region. Occasionally an apical five chamber view was used to align more parallel to the outflow tract and aorta. Sample

volume position: In the left ventricle just proximal to the aortic valve and midway between the septum and open anterior mitral valve leaflet (Figure 1).

Pulmonary Valve Flow

Imaging plane: Right parasternal short axis view of the right ventricular outflow tract at the aortic valve level. Sample volume position: In the pulmonary artery just distal to the valve (Figure 1).

Right Ventricular Outflow Tract

Imaging plane: Right parasternal short axis view of the right ventricular outflow tract at the aortic valve level. Sample volume position: In the right ventricle just proximal to the pulmonary valve and midway between the aortic wall and right ventricular wall (Figure 1).

3. Results and Discussion

3.1. Mitral Valve Flow

The mean + standard error and range of mitral valve flow were presented in Table 1.

Table 1: *Normal Pulsed Wave Doppler Velocities (n=20)*

Area	Mean + SE	Range
Mitral Valve Flow		
Peak E Velocity	69±2.60	50-90
Peak A Velocity	50±3.20	30-70
Ratio E:A	1.43±0.05	1.14-2.0
Left Atrial Flow		
Peak E Velocity	56.5±2.56	40-80
Peak A Velocity	36.5±2.35	25-60
Tricuspid Flow		
Peak E Velocity	58.5±3.20	40-90
Peak A Velocity	40.0±3.12	30-70
Ratio E:A	1.40±0.04	1.16-1.71
Right Atrial Flow		
Peak E Velocity	49±3.43	30-85
Peak A Velocity	35.5±3.42	20-75
Aortic Valve Flow		
Peak Systolic Velocity	119±4.28	100-160
Pulmonary Valve Flow		
Peak Systolic Velocity	103±5.03	70-140
Left Ventricular Outflow Tract		
Peak Systolic Velocity	103.5±4.35	75-140
Right Ventricular Outflow Tract		
Peak Systolic Velocity	91.5±4.47	60-130

During diastole the flow was positive and laminar with the two main phases to the flow each with a spike triangular appearance. The initial peak occurred during the rapid filling phase of early diastole with E being the point of peak velocity and the average E point velocity was 69±2.60 cm/sec with a range of 50-90 cm/sec. The second and usually smaller peak occurred in late diastole as a result of atrial contraction with A being the point of peak velocity. The average A point velocity was 50±3.20cm/sec with a range of 30-70 cm/sec. The mean E:A ratio was 1.43±0.05 with a range of 1.14-2.0 (Figure 2).

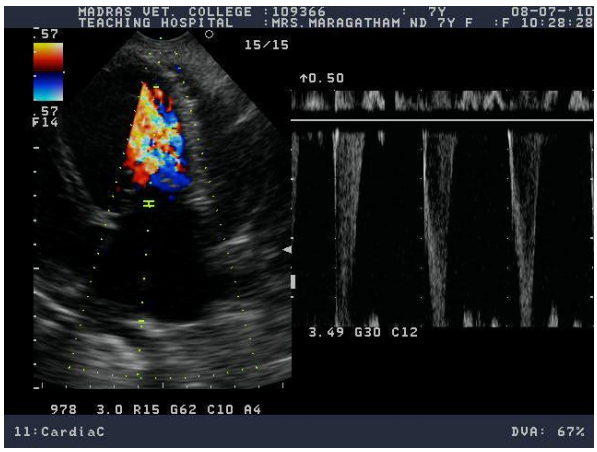

Figure 2: *Pulse wave Doppler measuring the mitral flow*

During systole a low velocity positive turbulent flow was found in 80 per cent of the dogs. In the present study the description of the wave form was in agreement with Kirberger et al., (1992). Peak E velocity was lower than the velocities reported by Belanger (2005) and Kirberger et al., (1992). Whereas peak A velocity was in agreement with Belanger (2005) who reported a range of 50-70 cm/sec. The reason behind the low peak E velocity may be the less number of dogs involved in the present study. In evaluating mitral and tricuspid valve flow, the origin of systolic positive turbulent flow signal is unclear. During early ventricular systole the arterial ventricular annulus move toward the cardiac apex (Tsakiris et al., 1971; Keren et al., 1986) and this movement of annulus toward sternum resulted in pushing the ventricle blood adjacent to the wall toward the transducer.

3.2. Left Atrial Flow

The mean ± standard error and range were presented in Table 1. During diastole the flow was positive and laminar and was similar to mitral valve flow. The peak velocity was slightly less than mitral flow velocity. The mean peak E velocity was 56.5±2.56 cm/sec with a range of 40-80 cm/sec and the mean A velocity was 36.5±2.35 cm/sec with a range of 25-60 cm/sec. During systole a low velocity turbulent flow found in 85 per cent of the dogs (Figure 3). Kirberger et al., (1992) reported that the mean peak E and A velocity as 76 cm/sec and 54 cm/sec respectively. This value was considerably higher than those reported in the present study. Similar to mitral valve flow less number of cases involved in this study may be quoted as a reason. The systolic positive flow in left atrium is probably caused by venous pulmonary inflow as described in man by Keren et al., (1986).

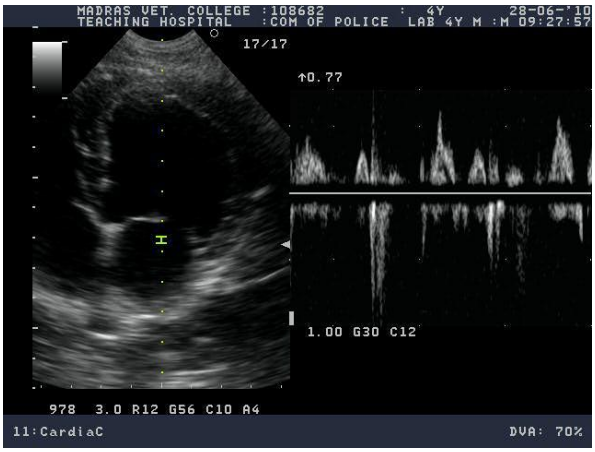

Figure 3: *Pulse wave Doppler measuring the left atrial flow*

3.3. Tricuspid Flow

The mean + standard error and range were presented in Table 1. The diastolic flow was similar to that of the mitral valve flow with an E and A point. The average peak E velocity was 58.5±3.20 cm/sec with a range of 40-80 cm/sec and the average peak A velocity was 40.0±3.12 cm/sec with a range of 25-60 cm/sec. Inspiration resulted in higher velocities especially in E peak. The average E:A ratio was 1.40±0.04 with a range of 1.16 – 1.71. During systole a low velocity positive turbulent flow similar to that described for mitral valve was found in 90 per cent of the dogs (Figure 4). Similar description of wave form were reported by Kirberger et al., (1992) but the average velocity of peak E and A was considerably lower in the present study which may be due to less number of dogs involved.

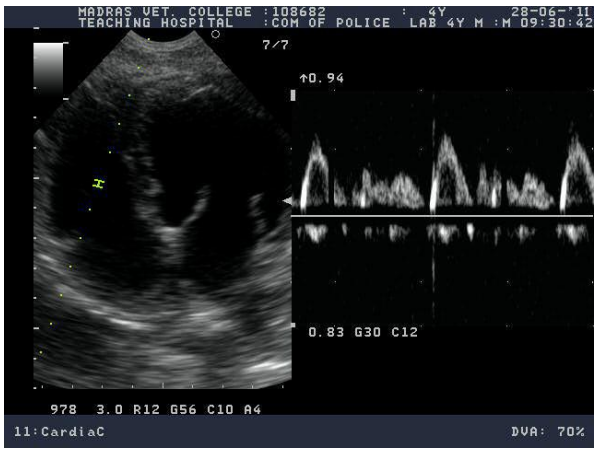

Figure 4: *Pulse wave doppler measuring the tricuspid flow*

3.4. Right Atrial Flow

The mean + standard error and range were presented in Table 1. During diastole the flow was positive and laminar and similar to tricuspid valve flow but the peak velocity flows were lower than tricuspid flow. Respiration had a definite effect on flow velocities, with inspiration resulting in higher velocities. The average peak E velocity was 49.0±3.43 cm/sec with a range of 30-85 cm/sec and peak A velocity was 35.5±3.42 with a range of 25-75 cm/sec (Figure 5). During systole a low velocity laminar to turbulent positive flow was found in 90 per cent of the dogs and the main contributor of this flow was believed to be systemic venous return. Observations made in the present study was similar to Kirberger et al., (1992) except that velocities peak E and A were lower. As earlier, less number of cases involved in the study may be assumed as the reason.

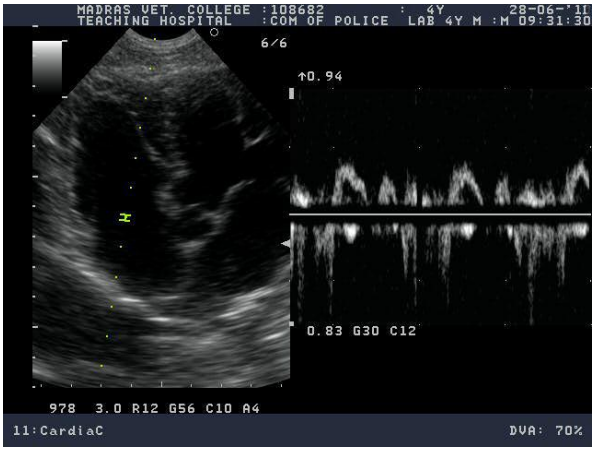

Figure 5: *Pulse wave doppler of tricuspid flow*

3.5. Aortic Flow

The mean + standard error and range were presented in Table 1. During systole the flow was negative and had a rapid laminar acceleration phase i.e. down stroke. Spectral broadening often started at the peak with the deceleration phase i.e. upstroke having widened spectrum. The rapid acceleration phase results in peak velocity being reached early in systole. The average peak velocity was 119±4.28 cm/sec with a range of 100-160 cm/sec. In 15 per cent of the cases, a negative low velocity turbulent flow was obtained during initial diastolic period. In the present study, the morphology of waveform is similar to findings of Kirberger et al., (1992). The present study was in full agreement with Yuill and O'Grady (1991) who reported the average peak velocity as 118 cm/sec.

3.6. Left Ventricular Flow

The mean + standard error and range were presented in Table 1. During systole the flow was negative and similar to aortic flow but with lower velocities. The initial acceleration phase was laminar with spectral broadening starting at the peak and continuing at the deceleration of the phase. Diastolic flow usually had positive and negative flow components with the degree of each depending on exactly where in the outflow tract the cursor was placed. The positive diastolic flow was similar to mitral valve flow being laminar and biphasic and starting at the beginning of diastole representing mitral flow toward the cardiac apex. The average peak velocity was 103.5±4.35 cm/sec with arrange of 75-140 cm/sec. The description of waveform in the present study is in agreement with Kirberger et al., (1992) but the peak systolic velocity recorded in the present study was considerably lower than reported by the above author. The left ventricular outflow tract diastolic flow was similar to that described in man (Panayiotou and Byrd, 1990). The positive diastolic flow probably represents passive mitral flow contaminating the sampling site and the negative diastolic flow probably represents mitral valve flow being deflected at the cardiac apex back towards the aortic flow.

3.7. Pulmonary Flow

The mean + standard error and range were presented in Table 1. During systole the flow was negative and the initial acceleration phase had a narrow spectral width but broadened slightly after the peak during the deceleration phase. Inspiration resulted in higher velocities. 25 per cent of dogs had low velocity negative diastolic flow which was late diastolic. The waveforms were similar in morphology to the finding reported by Kirberger et al., (1992). The peak systolic velocity was 103±5.03 cm/sec with a range of 72-100 cm/sec. The peak systolic velocity reported by Yuill and O-Gready was 96 cm/sec and this finding is in partial agreement with the present study.

3.8. Right Ventricular Outflow Tract

The mean + standard error and range were presented in Table 1. During systole the flow was negative and similar to pulmonary artery flow but with low velocities. A positive diastolic flow was recorded in 60 per cent of dogs and negative diastolic flow was recorded in 70 per cent of dogs. The average peak systolic velocity was 91.5±4.47 cm/sec with a range of 60-130 cm/sec. Except for the velocity the wave pattern were similar to finding by Kirberger et al., (1992).

3.9. Normal Color Flow Doppler Studies

Mitral and Tricuspid Flow

Typical left apical four chamber view showed mitral flow as a red with brighter central area and when Nyquist limit was exceed the central area of flow may have layers of blue superimposed on it. The same was recorded for tricuspid flows. The red color flow was due to the direction of Red blood cells

towards the transducer and when the nyquist was exceded the flow was layered with blue. Finding in the present study is in agreement with Boon (1998) and Kirberger et al., (1992).

Aortic Flow

Aortic flow was seen as hues of blue and red as blood leaves the left ventricle in a downward direction in the left apical five chamber view. The depth of aorta in this view resulted in a low Nyquist limit and wraparound and aliasing was seen. During early systole when the velocities were high, aortic flow showed an almost completely aliased signal, later in systole during deceleration much of the flow was accurately mapped as blue. The present study is in agreement with the color flow pattern reported by Boon (1998).

Pulmonary Flow

Pulmonary artery flow encoded on the transverse image of heart at the base in right parasternal short axis view was usually blue as blood leaves the right ventricle in a direction away from the transducer. Since the artery curves, this plane had a layering of color within the pulmonary artery profile. Total reversal of colors was often seen as flow progress from high velocity during early systole to slower velocities at the end of systole. The present study was agreement with Boon (1998).

3.10. Pulsed Wave Doppler and Color Flow Doppler Studies in Mitral Valvular Insufficiency and Dilated Cardiomyopathy

Mitral Valvular Insufficiency

The mean + SE and Range were presented in the Table 2. The average peak E velocity of the mitral valve, average peak systolic velocity of aortic valve and pulmonary valve were 122.5±5.43, 108.5±4.15 and 95.5±3.76 respectively. A highly significant increase in the peak E velocity of mitral valve was observed in the dogs with mitral valve insufficiency when compared to normal dogs. The increased E velocity may be due to increased atrial pressure or volume associated with mitral insufficiency. There was no significant difference in dogs with mitral valve insufficiency in aortic and pulmonary valve velocities. This indicates that the cardiac output is preserved and may be majority of dogs taken up for study was not having systolic failure.

In the present study attempt was made to measure the regurgitant jet velocity in left atrial side. Since only pulsed wave Doppler was used for this study, invariably all the dogs showed a velocity of more than 300 cm/sec beyond which it was not possible to measure. Kienele and Thomas (1995) reported that the peak velocity of mitral regurgitant flow as 500 cm/sec regardless of the magnitude of the regurgitant fraction and he also opined that if the peak velocity was less than 450 cm/sec, improper technique, very high left atrial pressure or misdiagnosis may be considered.

Color flow Doppler study of mitral regurgitation revealed that two out of ten dogs had jets occupying less than 20 per cent of atrium, three out of ten dogs had jets occupying 20-50 per cent of atrial area and five out of ten dogs had jets occupying more than 50 per cent of atrium and these jets were visualized in the atrium as a aliased regurgitant flow having a typical mosaic pattern.

Table 2: *Pulsed Wave Doppler Studies in Mitral Valvular Insufficiency and Dilated Cardiomyopathy*

Mitral Valvular insufficiency			
Area	**Normal**	**Mitral valvular insufficiency**	**F value**
Mitral valve flow			
Peak E systole	69 [a] ±2.60	122.5 [b] ±5.49	00.95**
Aortic valve flow			

Peak systole	119 [a] ±4.28	108.5 [a] ±4.15	2.410NS
Pulmonary valve			
Peak systole	103 [a] ±5.03	95.5 [a] ±3.76	0.96NS
Dilated Cardiomyopathy			
Area	**Normal**	**Dilated cardiomyopathy**	**F value**
Mitral valve			
Peak E velocity	69 [a] ±2.60	99.5 [b] ±4.24	41.3**
Tricuspid valve			
Peak E velocity	58.5 [a] ±2.56	55 [a] ±3.16	0.47NS
Aortic valve			
Peak systolic Velocity	119 [a] ±4.28	68 [b] ±5.28	51.16**
Pulmonary valve			
Peak systolic velocity	103 [a] ±5.03	61.5 [b] ±5.53	25.98**

**** -** Highly Significant
NS – Not Significant
Mean values bearing the same superscript in a same row do not differ significantly

Boon (1998) graded insufficiency as mild, moderate and severe based on the jets occupying the area of left atrium. He suggested that a jet that occupies less than 20 per cent of the atrium may be considered to represent mild insufficiency and that occupies 20-50 per cent of atrial area as moderate insufficiency and greater than 50 per cent as severe insufficiency. Muzzi et al., (2003) in a study on 61 dogs with mitral regurgitation opined that the assessment of regurgitation jet area by color flow Doppler mapping was useful in accurate identification and semiquantitative estimation of the severity of mitral regurgitation.

Dilated Cardiomyopathy

The average E velocity of mitral valve and tricuspid valve affected in dilated cardiomyopathy were 99.5±4.24 and 55±3.16. The peak systolic velocity of aortic and pulmonary valve flow in dogs with dilated cardiomyopathy was 68±5.28 and 61.5±5.53. A highly significant increase in the peak E velocity of mitral valve was observed in dogs with dilated cardiomyopathy compared to normal whereas no significant difference was appreciated between normal dogs and dogs with dilated cardiomyopathy in peak E velocity of tricuspid flow.

A highly significant decrease in the peak systolic velocity of aortic valve flow and pulmonary valve flow was observed in dogs with dilated cardiomyopathy compared to normal. Boon (1998) appreciated increased E velocity in dogs with dilated cardiomyopathy and he attributed this to the increase in atrial pressure or volume associated with secondary mitral insufficiency. He also recorded low aortic and pulmonary velocities in dogs with dilated cardiomyopathy which may be due to systolic failure.

In the present study four out of ten dogs showed both mitral and tricuspid regurgitation which was appreciated as aliased regurgitant flow having a typical mosaic pattern but the jets were occupying less than 50 per cent of atrial area which indicated that it was only a mild to moderate insufficiency. Two out of ten dogs showed mitral regurgitation alone indicating more involvement of left side. The regurgitation appreciated in dilated cardiomyopathy was due to abnormal dilation of chamber and consequent leak in Atrioventricular valve.

4. Conclusion

From the above study it was concluded that pulsed wave Doppler was very useful in recording flow velocities at all the valvular levels in a noninvasive fashion, which makes this diagnostic aid as a less cumbersome and more reliable technique, Color flow Doppler studies covers a larger area of flow and was instantaneous and effective in diagnosing flow abnormalities especially in mitral valve disease. It was also helpful in grading the severity as mild, moderate and severe whereas its use in Dilated

Cardiomyopathy was much limited. Therefore, colour Flow Doppler may be a real boon for an inexperienced personnel.

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Research Article **Open Access**

Exploration of the Physio-Chemical Properties of *Kedrostis foetidissima (Jacq.) Cogn.* Herb by Proximate and Qualitative Phytochemical Analysis

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Abstract In a herb, presence of phytochemicals and its physiochemical properties are responsible for its pharmacological and therapeutic potentials. *Kedrostis foetidissima (Jacq.) Cogn.* is an herbaceous perennial plant belongs to Cucurbitaceae family, having worldwide distribution and still being used traditionally for many therapeutic purposes. Proximate analysis and preliminary qualitative phytochemical analysis of this plant was carried out in this study to explore the physical and chemical characters like moisture, crude protein, crude fibre, total ash, ether extract, sand / silica, salt, calcium, phosphorus, gross energy and phyto-constituents which are reasons for the plant having different pharmacological activities such as antimicrobial, immunomodulatory, anti-inflammatory, antioxidant and growth promotion.
Keywords *Phytochemical Evaluation; Proximate Analysis; Kedrostis foetidissima (Jacq.) Cogn.*

1. Introduction

Kedrostis foetidissima (Jacq.) Cogn. commonly known as Appakovai in Tamil is a medicinal plant. It has the habitat of growing in rain-forest, river margins, deciduous and semi-evergreen woodland, dry bush lands, wooded grassland and around the fence. The distribution is worldwide and rich in the regions of South Africa and Asia. In India, the *Kedrostis foetidissima (Jacq.) Cogn.* is widely distributed in the areas of Gujarat, Punjab, Uttar Pradesh, Maharashtra, Tamilnadu and Andhra.

The herb is a perennial plant producing annual stems up to 3 meters long from a tuberous, perennial rootstock. The leaves have a very unpleasant smell, but a favored food of some native peoples, where they are commonly harvested from the wild for local use (Leffers, 2003). This herb found to be very effective in the treatment of asthma, urinary tract infections, diarrhoea and skin diseases. The

leaf extracts were used as anti-fouling agents especially for the treatment of bloat in cattle. The leaf juice was used for the treatment of cold in children and used as immune modulator (Nirmala and Pandian, 2013). The whole plant extracts of *Kedrostis foetidissima (Jacq.) Cogn.* was taken orally for curing chest pain in humans and the leaves were used in traditional ethnoveterinary medicine for the treatment of Aloye disease in cattle by the Zay people in Ethiopia (Giday et al., 2003). The decoction was used to treat diarrhoea in babies of 3 - 4 months of age in Uganda, (Tabuti *et al.,* 2003) and India (Amutha and Lalitha, 2012). The Paliyan tribes in Tamilnadu used the juice of the leaves of *Kedrostis foetidissima (Jacq.) Cogn.,* herbs for the treatment of common cold, cough and asthma in children (Karuppusamy, 2007). The indigenous people lived in the rain forest of Africa and South America consumed *Kedrostis foetidissima (Jacq.) Cogn.,* leaves as edible food in boiled form as snacks at times of food scarcity (Teklehaymanot and Giday, 2010).

Proximate and nutrient analysis of herbs plays a crucial role in assessing nutritional significance and health effects (Taiga, 2008). WHO has also emphasized on the importance and need of determining proximate analysis on herbal drug's standardization (Niranjan and Kanaki, 2008). The medicinal and therapeutic potentials of herbs depend upon the type of phytochemical substance they synthesize and store. In a novel drug discovery, the basic and essential details regarding the chemical constituents are generally provided by the qualitative phytochemical screening of plant extracts (Das Talukdar *et al.,* 2010). Hence the present study was taken to estimate the physiochemical and phytochemical properties of *Kedrostis foetidissima (Jacq.) Cogn.,* for future exploration of its pharmacological and therapeutic potentials.

2. Materials and Methods

2.1. Collection and Identification of Plants

Whole plant of *Kedrostis foetidissima (Jacq.) Cogn.,* was collected from base villages of Siruvani hills, Coimbatore, Tamilnadu, during the period of July to August and authenticated by the Department of Botany, Arignar Anna Government Arts and Science College, Namakkal, Tamilnadu, India.

2.2. Preparation of Crude Powder

The collected plants were washed with pure water and blotted gently on filter paper sheets and shade dried. The plant materials were finely powdered using a mechanical mixer / grinder after complete drying. The whole plant powder was used for proximate analysis and for the preparation of aqueous and alcoholic extracts.

2.3. Composite Analysis of Herbal Powders

100 g crude powders of *Kedrostis foetidissima (Jacq.) Cogn* whole plant was used for composite analysis to estimate moisture, crude protein, crude fibre, total ash, ether extract, sand & silica, salt, calcium, phosphorus and gross energy at Animal Feed Analytical and Quality Assurance Laboratory (AFAQAL), Veterinary College and Research Institute, Namakkal, Tamilnadu by using standard composite analysis procedures.

2.4. Preparation of Aqueous and Alcoholic Extracts

Aqueous and alcoholic extracts were prepared separately from the powdered plant materials of 100 g each by using 400 ml of sterile distilled water and 400 ml of ethanol respectively. Both extracts were kept in an orbital shaker for 48 hours at room temperature. Then the extracts were filtered by using Whatman filter paper No. 1, to separate the extractable substances. The collected filtrate were then evaporated at 37°C on hot air oven and the dried extracts were collected in a sterile container and

stored at 4°C, until used for study.

2.5. Qualitative Phytochemical Analysis

The qualitative phytochemical analysis of aqueous and alcoholic extracts of *Kedrostis foetidissima (Jacq.) Cogn (*whole plant) was done by using the method of Trease and Evans (1983) and Kokate et *al.,* (1990) at the laboratory of Ethno Veterinary Herbal Research Centre for Poultry, Teaching Veterinary Clinical Complex Campus, Namakkal, Tamilnadu.

5 gm of dried aqueous and alcoholic extracts of both the plants were added with 50 ml of distilled water and heated below 50^{o}C for 1-2 minutes and utilized for the detection of various phytochemicals.

3. Results and Discussion

3.1. Proximate Analysis of Herbs

The proximate analysis result of whole plant crude powder of *Kedrostis foetidissima (Jacq.) Cogn.,* showed a variant proportion of constituents depicted in Table 1. The *Kedrostis foetidissima (Jacq.) Cogn.,* showed significant levels of calcium, phosphorus, crude protein, total ash and gross energy.

Table 1: *Proximate analysis of whole plant powder of Kedrostis foetidissima (Jacq.) Cogn.*

S. No.	Constituents	level
1	Moisture (%)	9.11
2	Crude Protein (%)	17.47
3	Crude Fibre (%)	16.19
4	Ether Extract (%)	3.70
5	Total Ash (%)	20.52
6	Sand & Silica (AIA) (%)	5.85
7	Calcium (%)	3.49
8	Phosphorus (%)	0.39
9	Salt (%)	0.89
10	Gross Energy (kcal/kg)	3305

3.2. Qualitative Phytochemical Analysis

The extractive value for the aqueous and alcoholic was calculated and was found to be 7.3 and 6.5% respectively. The extracts were further examined for its physical characterization like color, odor, consistency etc. The color of the aqueous extract was brownish green with crystalline sediments on drying. The colour of the alcoholic extract was darkish green with semi solid oily consistency on drying. Both the extracts had characteristic odor, showed the suspect of having various phytochemicals.

Phytochemicals and its secondary metabolites are responsible for their pharmacological and therapeutic actions like antimicrobial, immunomodulatory, anti-inflammatory, antioxidant, antiviral, wound healing, anticancer, anthelmintic and growth promotion (Erdman *et al.,* 2007). The qualitative phytochemical screening of aqueous and alcoholic extracts of whole plant crude powder of *Kedrostis foetidissima (Jacq.) Cogn.* is showed in Table 2. The alcoholic extract phytochemical analysis results of *Kedrostis foetidissima (Jacq.) Cogn.* indicates the presence of various constituents like saponins, phenols, tannins, alkaloids, flavonoids, triterpenoids, volatile acids and glycosides in moderate to good amounts and in low amounts of phylobatannins and hydrolysable tannins. The aqueous extracts showed the same, but in low level and with the absence of phenols, phylobatannins, volatile acids, hydrolysable tannins and glycosides. Presence of terpenoids, saponins, phenols, tannins, alkaloids,

volatile acids, glycosides and flavonoids in the ethanolic extract of *Kedrostis foetidissima (Jacq.) Cogn.,* showed promising suspect on the herb for having various pharmacological potentials.

Table 2: *Phytochemical screening of aqueous and alcoholic extracts of whole plant of Kedrostis foetidissima (Jacq.) Cogn.*

S. No.	Phytochemicals	Extract	
		Aqueous	Alcoholic
1	Saponins	+	++
2	Tannins	+	++
3	Phenols	-	++
4	Alkaloids	+	++
5	Terpenoids	+	+++
6	Flavonoids	+	++
7	Amino acids and Proteins	-	-
8	Carbohydrates	-	-
9	Phylobatannins	-	+
10	Volatile Acids	-	++
11	Hydrolysable tannins	-	+
12	Glycosides	-	++
13	Cardiac Glycosides	-	-
14	Vitamin C	-	-

4. Conclusion

Significant level of crude protein, calcium, gross energy in the whole plant powder of *Kedrostis foetidissima (Jacq.) Cogn.*, and presence of various therapeutic phytochemicals in the ethanolic extract, subjected the herb for further detailed phytochemical studies and search for promising pharmacological potentials in lab animal, animal and poultry models.

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Research Article **Open Access**

Whether Peripartum Nutritional Supplementation Influence the Uterine Involution and Postpartum Fertility in Crossbred Cows

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Abstract The aim of this study was to investigate the effect of peripartum nutrients supplementation on uterine involution and postpartum fertility in crossbred cows. Twenty healthy advanced pregnant HF crossbred cows between 2-4 parity of identical body size were included in the study from two weeks prepartum to 8 weeks postpartum. They were equally divided into control (routine farm feeding-RFF) and treatment (RFF + bypass fat @ 100-200 g/h/day + ASMM @ 50 g/h/day) groups. Animals of both the groups were subjected to per rectal as well as ultrasonographic examinations at weekly intervals from day 7 till day 42 postpartum. On day 7 postpartum, gravid and non-gravid uterine horns in all animals were lying cranial and ventral to pelvic brim in abdominal cavity as large, soft, flabby water bag like structures. The cervical and uterine horn diameters and wall thickness showed a significant decreasing trend from day 7 to 28-35 postpartum with gaining tonicity and elasticity, yet the differences between groups were non-significant. The walls of the cervix and uterus appeared as bright hyperechoic structure, while lumens were found hypoechoic with bright hyperechoic spots. The middle uterine arteries were represented as dark circular anechoic structures and the caruncles as a bright hyperechoic structures. The diameter of artery reduced significantly ($p<0.05$) in the days postpartum. The gross involution of the uterus was observed to be completed by 24.50±1.14 and 23.80±1.14 days, while the complete uterine involution occurred by days 31.97±1.82 and 30.27±1.41 with occurrence of first estrus postpartum in 42.32±4.14 and 38.00±1.95 days ($p<0.05$) for control and nutrient supplemented groups, respectively. The service period (85 vs 100 days) and pregnancy rate (80 vs 60%) were non-significantly better in treatment than control group. Thus it can be asserted that the peripartum nutrient supplementation yielded no beneficial effect on uterine involution, but enhanced postpartum fertility in optimally fed and managed crossbred cows.
Keywords *Crossbred Cows; Nutritional Supplement; Uterine Involution; Clinical & Ultrasound Monitoring*

1. Introduction

The main factors which contribute to economic losses in dairy animal entrepreneur are delayed uterine involution, longer calving interval and short productive life. Fats in the diet can influence

reproduction positively by altering both ovarian follicle and corpus luteum function via improved energy status and by increasing precursors for the synthesis of reproductive hormones such as steroids and prostaglandins. Factors such as limited energy intake, lower body reserves and postpartum diseases can delay the uterine involution and thereby the ovarian recrudescence may also be hampered. The greatest change in the uterus occurs within a few days postpartum. Uterine involution and diameter of uterine horns can be monitored directly by palpation per rectum (Suthar and Kavani, 1992; Kindahl et al., 1999) or by using the transrectal ultrasonography (Sheldon et al., 2004; Parikh, 2009). Therefore, this investigation was planned to study the effect of incorporation of chelated minerals as well as bypass fat in the ration of transitional crossbred cows on their uterine involution and postpartum fertility.

2. Materials and Methods

2.1. Selection of Animals

The study was carried out at University Farm during November 2014 to May 2015 on 20 advanced pregnant HF crossbred cows of 2nd to 4th parity and of nearly identical body size from two weeks prepartum to 8 weeks postpartum. All the pregnant cows were maintained in well ventilated hygienic sheds and were stall fed as per feeding schedule followed on the farm, and had free access to drinking water. The term cows were isolated 60 days before calving in a separate shed. The cows approaching parturition were segregated in calving pen to monitor the calving events and separate the new born calves as the farm follows weaning system. The freshened cows were transferred to the lactating group of cows on the next day of normal calving and were machine milked twice daily.

2.2. Experimental Groups

The selected 20 cows were randomly and equally divided into two group, control and treatment and were managed as under.

Control Group (n=10): These cows were maintained on routine farm feeding schedule (green fodder, hay and compound concentrate mixture @ 18-20, 4-5 and 3.0-3.5 kg, respectively, with 50 g of mineral mixture, Amul brand) during last two months of pregnancy and postpartum. After calving the level of compound concentrate fed was @ 40 per cent of milk produced.

Treatment Group (n=10): In addition to routine farm feeding, these cows were supplemented daily with extra 50 g of area-specific multi-minerals (developed by ANRS, AAU) and 100 g of bypass fat (Sunegry, Polchem) with compound concentrate mixture (Amul dan) for 2 weeks each before and after calving. The level of bypass fat was then increased as per the milk production @ 15 g per litre of milk produced until 60 days postpartum limiting to maximum of 200 g/day.

2.3. Clinical and Ultrasonographic Examinations

Per-rectal (using precalibrated hand) as well as ultrasonographic (using 5-10 MHz B mode transrectal transducer) evaluations of uterine and cervical involution were carried out along with monitoring of ovarian activity at weekly interval from day 7 till 42 postpartum as per Dobson-Hill (2009). All the animals were followed till 90 days postpartum for puerperal events and occurrence of postpartum first and fertile estrus. Animals detected in estrus after 60 days of calving were inseminated. For uterine involution following criteria were considered by giving the non-parametric scores to the parameters as per Scully et al. (2013). Uterine position was scored on a 0-3 scale [0= uterus and uterine horn returned to the previously non-gravid state and 1-3= uterine body and horns falling further over the pelvic brim i.e., between the pelvic brim and abdominal cavity], Size (Comparative size; >cervix 1; =

cervix 2; <cervix 3) of cervix, gravid uterine horn and non-gravid uterine horn, and Tone and consistency (low tonicity 1; moderate tonicity 2; good tonicity 3).

Cows were scanned trans-rectally using a linear array transducer, which was positioned dorsally and parallel over the uterus and sound beams were directed dorso-ventrally. Approximately 10 cm ahead from the gross bifurcation, the uterine diameter and thickness of uterine wall were measured as per the guidelines of Melendez et al. (2004) for both gravid and non-gravid horns. Two diameters of horns were evaluated; the first one from serosa to serosa to obtain the outer diameter of the uterine horn (Sheldon and Dobson, 2000) and the second from mucosa to mucosa to obtain the lumen diameter (Melendez et al., 2004). The difference between the first and the second measurements was divided by 2 to arrive at the estimate of the thickness of the uterine wall (Parikh, 2009). The diameters of middle uterine arteries of either side were measured by keeping the transducer transversely. Caruncles located approximately at the same position of scanning for uterus were measured for its length and width. The uterine and cervical involution in terms of diameters on different days and total time in animals of control and treatment groups were compared using ANOVA and 't' test.

3. Results and Discussion

3.1. Clinical Evaluation of Uterine Involution

On day 7 postpartum, gravid and non-gravid uterine horns were lying cranial and ventral to pelvic brim in abdominal cavity as large, soft, flabby water bag like structures without tonicity and elasticity in all the animals irrespective of groups and only non-gravid horn could be palpated to its full length. Morrow et al. (1969) also reported that the uterine horns were palpable cranial and ventral to the pelvis at days 4 to 7 postpartum in dairy cattle. By day 14 postpartum, the non-gravid horn and the cervix were found to be nearer to the pelvic brim than the gravid horn. By the end of the third, fourth and fifth week, the trend for position scores indicated that gravid horn involuted later as compared to the non-gravid horn. Practically, by the end of 6^{th} week (42 days postpartum), the gravid and non-gravid horns as well as cervix were found to be located in the pelvic cavity with previous non-gravid state and concurred with Scully et al. (2013). Thus, based on the position scores, the uterine involution was observed to be completed by days 35 postpartum in all the animals with no apparent effect of nutrients supplementation.

By day 21 postpartum, both the gravid as well as non-gravid horns had their size comparatively smaller than the cervix in all 10 cows of treatment group, and in 9/10 animals of control group, and 70 and 80 % cows of control and treatment groups, respectively, had their genitalia located within the pelvic cavity. The remaining cows had their genitalia within the pelvis by 28^{th} day postpartum and the horns appeared to be symmetrical. The uterine horns were palpated as smaller in size when compared to the cervix from 21 days onwards up to 42 days postpartum. These findings were supported by the observations of Gier and Marion (1968), that till about 25 days postpartum, the diameter of the uterus exceeded the diameter of the cervix.

The gross involution of the genitalia was observed to be completed approximately by 24.50±1.14 days in all the cows under control group, whereas the corresponding time taken by the cows under treatment group was 23.80±1.14 days. The increasing trend in the tonicity, elasticity and curling of horns was observed from day 7 postpartum to days 21 or 28 postpartum in all the animals with the highest score 3 on day 21 and 28 postpartum, being more pronounced in treatment group. On day 42 postpartum, the tonicity, elasticity and curling of the uterine horns were observed to be increased further as observed by Sutaria (2010) in Kankrej cows.

The observed reduction in size of organs and gradual increase in tonicity of genitalia from the day 7 to 42 postpartum in all animals under both the groups were in accordance with reports of Gier and

Marion (1968) and Jadhav (2005). However, Roberts (1971) opined that an increase in uterine tone occurs from days 10 to 14 coinciding with the onset of first postpartum estrus. The decrease in size of uterine horns was observed to be faster up to day 14 to 21 postpartum and thereafter it decreased very marginally in the present study as noted by Tennant et al. (1967) and Sutaria (2010). The present findings of gross uterine involution (23.80 to 24.50 days) observed are in close agreement with the earlier results of several researchers (Tiwari, 1999; Patel et al., 2005; Sutaria, 2010; Saut et al., 2011). The mean time recorded for uterine involution was, however, found to be comparatively lower than the values (30 to 45 days) reported by Guilbault et al. (1987), Sattar et al. (2007), Heppelmann et al. (2013) and Scully et al. (2013).

3.2. Ultrasonographic Evaluation of Uterine Involution

(a) *Echogenicity of Reproductive Tract*

The wall of the cervix appeared as bright hyperechoic structure, while its lumen was found to be hypoechoic with bright hyperechoic spots of cervical folds as recorded by Sutaria (2010) in Kankrej cattle. By day 7, the cervix was found to be reduced in diameter considerably with constant reformation of the folds evinced through echogenecity. The echogenicity of the cervix became even more pronounced with the hyperechoic cervical wall and the cervical folds being maintained in subsequent days postpartum. These findings were in accordance with the observations of Wehrend et al. (2003). Upon sonography of the uterine horns on the 7th day postpartum, the wall showed hyperechoic structures and the lumen was anechoic with some hyperechoic spots (Plate-I). On the later stages, the horns were seen as hyperechoic wall and hypoechoic lumen (Plate-II). By 42 days postpartum, the echogenicity of the uterine horns was much pronounced revealing the wall to be hyperechoic, whereas the lumen was hypoechoic (Plate-III). These findings concurred well with Gulvane (2005). The snowy appearance of the uterine lumen filled with lochia mixed with necrotic tissue debris was because of the differences in their echogenicity (Kamimura et al., 1993).

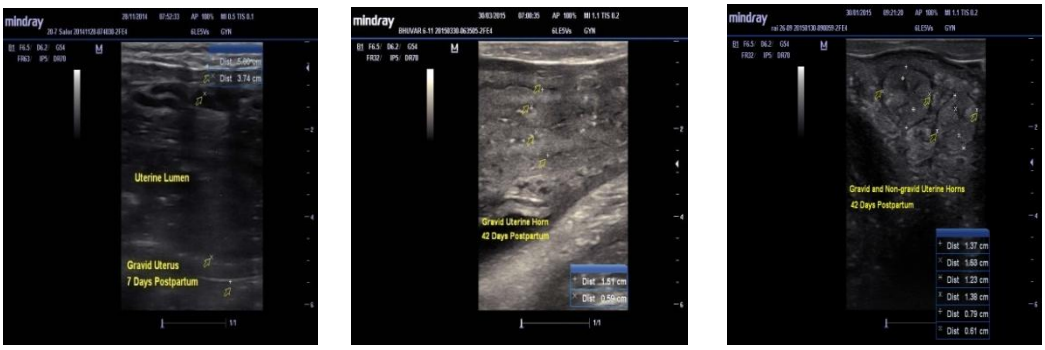

Plate I, II & III: *Ultrasonogram of Gravid uterine horn on day 7 and 42 postpartum*

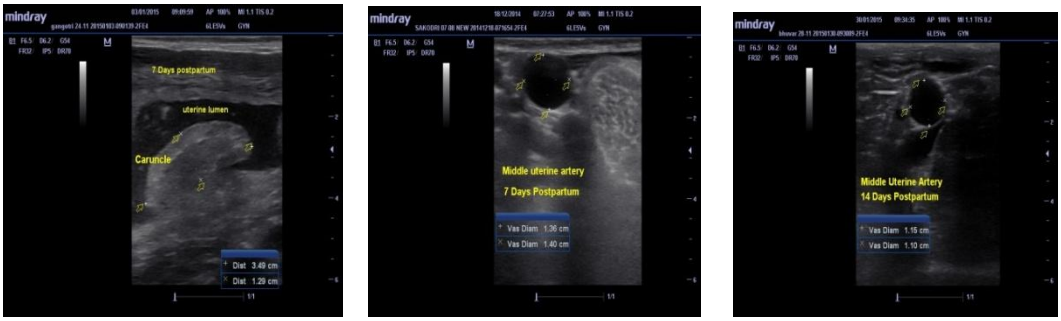

Plate IV, V & VI: *Ultrasonogram of Maternal caruncle and Middle uterine artery on day 7 & 14 postpartum*

The scanned uterine caruncles on day 7 postpartum were represented as a bright hyperechoic structures resembling mushroom, protruding in the anechoic uterine lumen, encircled with bright visible hyperechoic line (Plate-IV). The texture of the caruncles was similar afterwards but highly reduced in dimensions, i.e., in length and width. These findings corroborated with the reports of Gulvane (2005) and Jadhav (2005). However, Sutaria (2010) observed the caruncles as bright hyperechoic structure having hypoechoic spots in Kankrej cows. The echogenicity of middle uterine artery did not differ from day 7 to 14 postpartum and appeared as a black circular structure (Plate-V, VI) as noted by Sutaria (2010) and Heppelmann et al. (2013).

(b) *Diameter of Cervix and Its Wall Thickness*

The cervical diameter and wall thickness measurements revealed a significant reducing trend ($p<0.01$) from day 7 to 28, and thereafter non-significantly till day 42 postpartum in both the groups (Table 1). The day-wise variation observed in the mean diameter of the cervix between the groups was however non-significant. These observations corroborated well with the findings of Gier and Marion (1968), Guibault et al. (1987), Jadhav (2005), Sutaria (2010) and Kaewlamun et al. (2011) in pure, exotic, zebu and/or crossbred cows. The trend of reduction in cervical diameter and wall thickness throughout postpartum period was physiologically normal and similar, irrespective of groups, and indicated that the cervical involution was completed by day 28 postpartum, yet the fact is that the involution process was still proceeding as the thickness of the cervix was reducing till 42 days postpartum. These findings were in close agreement with Wehrend et al. (2003), Kasimanickam et al. (2004) and Sutaria (2010).

Table 1: *Mean ultrasonographic measurements of cervical diameter and wall thickness in postpartum cows under control and nutrient supplemented (treatment) groups*

Days post-partum	Cervical diameter (cm)		Pooled mean (n=20)	Cervical wall thickness (cm)		Pooled mean (n=20)
	Control (n=10)	Treatment (n=10)		Control (n=10)	Treatment (n=10)	
7	5.13±0.05^{d}	5.44±0.10^{d}	5.28±0.07	0.79±0.02^{e}	0.82±0.02^{d}	0.81±0.02
14	4.48±0.14^{c}	4.53±0.11^{c}	4.50±0.12	0.70±0.03^{d}	0.68±0.01^{c}	0.69±0.02
21	3.51±0.16^{b}	3.82±0.11^{b}	3.66±0.13	0.60±0.03^{c}	0.56±0.05^{b}	0.58±0.04
28	3.15±0.02^{a}	3.19±0.04^{a}	3.17±0.03	0.52±0.02^{b}	0.52±0.03^{b}	0.52±0.04
35	2.99±0.08^{a}	3.09±0.04^{a}	3.04±0.06	0.43±0.01^{a}	0.43±0.01^{a}	0.43±0.01
42	2.88±0.09^{a}	3.06±0.03^{a}	2.97±0.06	0.43±0.01^{a}	0.40±0.01^{a}	0.42±0.01

The means bearing different superscripts within column differ significantly ($p<0.01$) between the time intervals

(c) *Diameter and Wall Thickness of Uterine Horns*

The mean diameter and wall thickness of gravid and non-gravid uterine horns of the crossbred cows under both the groups reduced significantly ($p<0.01$) and progressively between days 7 and 28, but thereafter, the reduction was non-significant. The apparent variations in the day-wise mean diameters and thickness of gravid as well as non-gravid uterine horns between groups were found to be statistically non-significant (Table 2, 3). The mean time interval for complete involution of cervix was recorded to be 40.60±0.93 days for control group and 37.80±1.14 days for treatment group, and the uterine involution was completed by 31.50±2.39 and 30.80±1.55 days in respective groups. The time required for complete involution was slightly lower in treatment group, however the difference was non-significant. Thus, it can be surmised that the animals supplemented with peripartum nutrients did not have any beneficial/contributory effect on genital involution.

Table 2: *Mean ultrasonographic measurements of gravid uterine horn diameter and wall thickness in crossbred cows under control and treatment groups*

Days post-partum	Gravid uterine horn diameter (cm)		Pooled mean (n=20)	Gravid uterine horn wall thickness (cm)		Pooled mean (n=20)
	Control (n=10)	Treatment (n=10)		Control (n=10)	Treatment (n=10)	
7	5.27±0.09^{d}	5.52±0.11^{d}	5.39±0.10	0.76±0.02^{d}	0.69±0.01^{d}	0.73±0.02
14	3.81±0.17^{c}	3.38±0.07^{c}	3.83±0.12	0.67±0.01^{c}	0.63±0.11^{c}	0.65±0.01
21	2.38±0.07^{b}	2.61±0.13^{b}	2.50±0.10	0.53±0.03^{b}	0.55±0.03^{b}	0.54±0.03
28	1.84±0.08^{a}	1.80±0.06^{a}	1.82±0.07	0.40±0.01^{a}	0.41±0.02^{a}	0.41±0.02
35	1.72±0.07^{a}	1.63±0.05^{a}	1.68±0.06	0.36±0.01^{a}	0.37±0.01^{a}	0.37±0.01
42	1.60±0.06^{a}	1.56±0.04^{a}	1.58±0.05	0.36±0.01^{a}	0.37±0.01^{a}	0.36±0.01

The means bearing different superscripts in column differ significantly ($p<0.01$) within the time intervals

Table 3: *Mean ultrasonographic measurements of non-gravid horn diameter and wall thickness in crossbred cows under control and treatment groups*

Days post-partum	Non-gravid uterine horn diameter (cm)		Pooled mean (n=20)	Non-gravid uterine horn thickness (cm)		Pooled mean (n=20)
	Control (n=10)	Treatment (n=10)		Control (n=10)	Treatment (n=10)	
7	4.27±0.08^{d}	4.47±0.11^{d}	4.37±0.10	0.71±0.01^{c}	0.65±0.01^{d}	0.68±0.01
14	3.18±0.11^{d}	3.26±0.02^{c}	3.22±0.07	0.67±0.01^{c}	0.56±0.01^{c}	0.62±0.01
21	2.18±0.07^{c}	2.51±0.13^{b}	2.35±0.10	0.51±0.03^{b}	0.51±0.02^{b}	0.51±0.02
28	1.81±0.08^{a}	1.76±0.07^{a}	1.79±0.07	0.38±0.01^{a}	0.40±0.02^{a}	0.39±0.01
35	1.69±0.07^{a}	1.63±0.05^{a}	1.66±0.06	0.36±0.01^{a}	0.37±0.01^{a}	0.36±0.01
42	1.60±0.07^{a}	1.55±0.03^{a}	1.58±0.05	0.30±0.01^{a}	0.36±0.00^{a}	0.35±0.01

The means bearing different superscripts in column differ significantly ($p<0.01$) within the time intervals

The uterine involution time recorded (31.62±1.62 days) through ultrasonography in the present study was in agreement with the findings (28-40 days) of Santos et al. (1994), Sheldon and Dobson (2000), Melendez et al. (2004), Zhang et al. (2010). However, it was slightly lower as compared to the values (40-45 days) reported by Kaewlamun et al. (2011), Heppelmann et al. (2013) and Scully et al. (2013). The present findings on the diameters of the uterine horns were comparatively lower than the findings of Jadhav (2005). The observed difference could be due to the breed variation. The reduction in the uterine wall thickness to complete uterine involution in our study was in agreement with the findings of Melendez et al. (2004) and Sutaria (2010). In the present study, the thicknesses of cervical and uterine walls continued to reduce for few more days even after the diameters of both the cervix and uterine horns were almost non-significantly different, indicating that the involution of cervix and uterus was not completed and it required few days more for complete involution as a mandatory change required for the next pregnancy to take place (Zemjanis, 1970).

(d) *Diameter of Middle Uterine Artery*

The mean diameters of middle uterine artery ipsilateral to the gravid uterine horn in cows of control group on day 7 and 14 postpartum were 1.31±0.03 and 1.08±0.02 cm, respectively, with the corresponding values of 1.33±0.02 and 1.13±0.03 cm in treatment group. Similarly, the values for artery of non-gravid side in cows of control group were 1.21±0.38 and 0.97±0.31 cm, and in treatment group 1.21±0.03 and 0.98±0.02 cm, respectively, the differences between days being significant ($p<0.01$) in both the groups for both the sides. The diminution of the middle uterine artery ipsilateral to both gravid and non-gravid horn was observed from day 7 to 14 postpartum for both the groups, with no significant difference between the groups. These findings concurred with the observations of Sutaria (2010) and Heppelmann et al. (2013), who also observed that the uterine artery on the non-gravid side reduced significantly ($p<0.05$) faster when compared to the gravid side.

(e) *Length and Width of Caruncles*

The mean caruncular lengths in animals of control group on day 7 and 14 postpartum were found to be 3.10±0.15 and 1.50±0.05 cm ($p<0.01$), respectively. The corresponding values in animals of treatment group were 3.13±0.13 and 1.29±0.04 cm, respectively, with significant difference ($p<0.01$). The corresponding mean caruncular widths in animals of control group were 1.23±0.03 and 0.52±0.03 cm, and in treatment group 1.23±0.03 and 0.53±0.04 cm, respectively. A trend of significant ($p<0.01$) regressive changes was observed in the caruncular length and width from day 7 to 14 postpartum in animals under both the groups. However, the day-wise differences between the groups were non-significant. These findings were in agreement with Roberts (1971), Jadhav (2005) and Sutaria (2010). They all reported that the dissolution and sloughing of the caruncles was generally completed by 12 days postpartum and returned to nearly their original size by 2nd to 3rd week postpartum.

3.3. Postpartum Fertility

The reduced time required for the expulsion of placenta (3.83±0.21 vs 4.81±0.65 h) and marginally lesser mean involution time (30.27±1.41 vs 31.97±1.82 days) obtained in the cows under treatment group than the control group, with significantly shorter mean interval of first postpartum estrus (38.00±1.95 vs. 42.32±4.14 days) and service period (85.22±7.17 vs 100.67±5.60 days) and higher pregnancy rate (80 vs 60%) might be due to the probable positive or beneficial effect of nutrient supplementation in the form of chelated minerals and bypass fat. These findings are in agreement with the reports of Kaewlamun et al. (2011) and Khalil et al. (2012), who found that there was no effect of nutritional and bypass fat supplementation on the process of uterine involution, but had beneficial effect on postpartum fertility. However, McNamara et al. (2003) and Tyagi et al. (2010) reported that nutrient supplementation in the form of bypass fat in the diet of the cows had positive effect on the rate of uterine involution.

The present findings clearly indicated that there was a positive effect of peripartum nutrient supplementation in the cows so far as onset of postpartum ovarian activity is concerned, and concurred with Tyagi et al. (2010), Khalil et al. (2012) and Aungier et al. (2014). The earlier resumption of cyclicity in the crossbred cows under treatment group could be attributed to the effect of supplementation in the diet. Fats in the diet influence reproduction positively by altering both ovarian follicle and CL function via improved energy status and by increasing precursors (insulin and IGF-I) for the synthesis of reproductive hormones such as steroids and prostaglandins. The increase in insulin (Palmquist and Moser, 1981) plays a role in mediating increased follicular growth, either directly through its own receptor or indirectly by modulating granulosa cell IGF-I production which is required for follicle development (Rahbar et al., 2014). The fatty acid supplemented in the present study also increased the blood glucose concentration which had a positive effect on preovulatory

follicles, by increase both in size and number (Lammoglia et al., 1997), with formation of larger corpora lutea having high progesterone values.

4. Conclusion

From the results of the study, it would be worth to surmise that the peripartum nutrient supplementation in the form of bypass fat and chelated minerals resulted into marginally shorter period of uterine involution, yet early onset of postpartum ovarian activity, with significantly ($p<0.05$) lower first postpartum estrus interval and service period as compared to control group of crossbred cows. Further, the regressive changes observed on per-rectal and ultrasonographic evaluation of genitalia reflected normal physiological puerperal events essential for new reproductive events in healthy dairy cows.

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Antisperm Antibodies in Blood Serum and Cervical Mucus of Cross-Bred Cows With Respect to Age, Parity and Number of Inseminations

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Abstract Role of antisperm antibodies in infertility of animals is still controversial. A study was planned with an objective to detect antisperm antibodies (ASA) by Immunoperoxidase assay (IPA), Sperm Mar test and ELISA in blood serum and cervical mucus of 53 cross-bred cows, which were interrelated with age, parity and number of inseminations. Cows were grouped according to age (years), parity- and insemination- number as G-1(< 3, 0 and 0), G-II (3.0 - 4.5, 1.0 and 1- 3), G-III (4.6 - 6.0, 2.0 and 4.0 – 6.0) and G-IV (> 6.0, ≥ 3.0 and > 6.0). In Sperm Mar test, 40% reaction between motile spermatozoa and coated latex particles of IgG and IgA is considered as lower limit of significant activity. Therefore, percentage of cows with higher level of ASA (> 40% IPA / IgG / IgA, 3200 - 6400 / 200 - 800 titre) was calculated in each group. Proportion of cows with higher serum-IgG and ELISA-titre in cervical mucus and that with higher ELISA titre in serum / cervical mucus increased with increased age and parity, respectively. According to number of inseminations, percentage of cows with higher level of ASA (IPA), IgG and ELISA titre in blood serum was maximum in G-IV, whereas that with IgA in G-III in comparison to other groups. Percentage of cows with higher level of ASA (IPA), IgG, IgA and ELISA in cervical mucus was higher in G-III as compared to other groups. This study exposed a significant increase in ASA in serum /cervical mucus of cross-bred cows only with increase in number of inseminations.
Keywords *ASA; Cross Bred Cattle; Age; Parity; Artificial Insemination*

1. Introduction

Role of anti-sperm antibodies (ASA) in infertility of animals is controversial. Studies done on prevalence of ASA by some authors revealed that ASA may inhibit fertility [1]. But other authors are of the opinion that ASA are irrelevant to infertility [2]. In females, the epithelium of the cervix is capable of producing a local immune response. Antibodies in cervical mucus or oviductal fluid could obstruct sperm transport or interfere with the capacitation / acrosome reaction [3, 4]. Immune responses vary from one animal to another and certain animals may have a genetic tendency for a more response. It has been suggested that if spermatozoa are present during an infection of the reproductive tract, the

infectious agents have an adjuvant effect and somehow potentiate the immune response against spermatozoa [3]. Development of cross-reacting antibodies (antibodies that are formed against one antigen but can also react with a second antigen) is another possibility, which may impair fertility in animals [5]. Occurrence of ASA in blood serum or CM of cows has been related to age [6], parity [6, 7], repeat breeding / number of inseminations and infertility [1, 7-11]. Therefore, ASA detected by Immunoperoxidase assay, Sperm Mar test and ELISA in blood serum (BS) and cervical mucus (CM) of cross-bred cows were interrelated with age, parity and number of inseminations.

2. Materials and Methods

2.1. Collection of Samples

Blood and cervical mucus were collected from 53 cross-bred cows from dairy farm, Guru Angad Dev Veterinary and Animal Sciences University and private dairy farms around Ludhiana city. Blood was collected in sterilized vials from jugular vein without anticoagulant, centrifuged at 3000 rpm for 5 min to separate serum. At the time of estrus, CM was collected with the help of AI pipette, sonicated at 20 watts, 3 X 20 secs. BS and CM were inactivated at 56^{o} C for 30 min and stored in aliquots at -20^{o} till further use. Data regarding age, parity and number of inseminations of 40, 40 and 53 cows could be obtained from the dairy farms at the time of sample collection.

2.2. Procurement of Semen

Frozen semen straws were procured from GADVASU, dairy farm.

2.3. Detection of Antisperm-Antibodies in Blood Serum and Cervical Mucus

2.3.1. *Immunoperoxidase assay* [IPA, 12]

Sperm smears on clean slides were incubated with 1% bovine sperm albumin for 2 hours at 4°C. Slides were washed thrice with PBS, pH 7.4, incubated with 1:200 diluted BS / 1:50 diluted CM for 1 hour at 37°C, again washed thrice with PBS. Smears were incubated with rabbit anti-bovine IgG (Sigma) for 45 minutes at 37°C and washed thrice with PBS. Colour was developed with 3, 3'-Diaminobenzindine tetrahydrochloride in Tris buffer (0.05M, pH 7.6 at 25°C) and 27µl of 3% hydrogen peroxide for 5 minutes at room temperature. Washed with distilled water, slides were mounted in 10% glycerol in PBS, covered with coverslip and examined under the microscope at 10 x 100 X for dark brownish colouration of the sperm. About 200 sperms with browning on acrosome, post acrosomal cap or whole head were counted in different fields and percentage of IPA positive sperms was calculated.

2.3.2. *Indirect Sperm Mar Test* [Sperm Mar kit, 13]

Diluted inactivated BS/CM 1/4 with TALP medium, pH, 7.4 and incubated at 37°C for 30 min. Collected the motile sperms by centrifugation through Histopaque, suspended the sperm pellet in TALP and adjusted the sperm conc. to 20 X 10^{6}. Incubated 100 µl of the sperm suspension of motile spermatozoa with 100 µl of inactivated ¼ diluted BS or CM, incubated for 1 hour at 37°C. Added 2 ml of TALP, mixed well and centrifuged for 10 minutes at 400 g. Re-suspended the pellet with 50 µl of TALP. On a slide, mixed 10 µl of sperm suspension and 5 µl of sperm Mart latex particles IgG / IgA, mixed, covered with cover slip, kept in humid chamber for 5 min and observed under microscope at 400 X. Attachment of latex particles to the head/ tail or whole sperm was observed. About 200 sperms in different fields were counted and percentage was calculated.

2.3.3. *Preparation of Sperm Antigen for ELISA*

Sperm antigen was prepared by suspending washed spermatozoa in 62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 1mM PMSF, 25 mM benzidine, 10mM aprotinin, 10mM pepstatin and 5mM soya bean trypsin inhibitor, sonicated (3 bursts of 20 sec each) and centrifuged at 15,000 rpm for 30 minutes.

2.3.4. *ELISA* [14]

ELISA plates were coated with 5 µg protein (sperm antigen) per well by incubating at 37ºC for three hrs. After washing thrice with PBS, antigen coating was blocked by incubating with 300 µl of 2% BSA per well for overnight at 4ºC. Again washed thrice with PBS pH 7.4 and added serial dilutions of BS/ CM into the wells and incubated at 37ºC for three hours. Washed again with PBS and incubated with 100 µl/well of HRP conjugated anti bovine IgG for three hours at 37ºC. Washed the plate twice with PBS and incubated with 100 µl of o-phenyldiamine + 0.06% H_2O_2 as a substrate for 20 min at room temperature. Stopped the reaction with 5 N H_2SO_4 and measured the absorbance at 492 nm using ELISA reader.

2.4. Statistical Analysis of Data

The data obtained was analyzed statistically according to Independent Sample T-Test and One-Way ANOVA using difference between means of two groups and means of different group application at 5 percent level of significance (SPSS, Version 16.0).

3. Results and Discussion

ASA were detected in all the tested cows irrespective of age, parity and number of inseminations. Cows were grouped according to age (years), parity and insemination (number) as G-1(< 3, 0, 0), G-II (3.0 - 4.5, 1, 1-3), G-III (4.6 - 6.0, 2, 4-6) and G-IV (>6, ≥3, >6). In Sperm Mar test [13], 40% reaction between motile spermatozoa and coated latex particles of IgG and IgA is considered as lower limit of significant activity. Therefore, percentage of cows with higher level of ASA (> 40% IPA / IgG / IgA, 3200 - 6400 / 200 - 800 titre) was calculated in each group. Table 1, 2 and 3 depicts the values of ASA in relation to age, parity and number of inseminations, respectively.

3.1. ASA in blood serum and cervical mucus of cows according to age (n=40)

ASA detected by IPA / Sperm Mar-IgG and -IgA were non-significantly ($p<0.05$) / significantly ($p>0.05$) and non-significantly ($p<0.05$) higher in BS of G-II and G-III, respectively (Table 1). ELISA titre in BS showed a significant ($p>0.05$) increase with increase in age. There was a non-significant ($p < 0.05$) higher percentage of IPA, IgG and IgA in CM of G-I, G-IV and G-I, respectively. ELISA- titre was also non-significantly (<0.05) higher in CM of G-IV as compared to G-I and G-III. The presence of circulating sperm antibody was significantly associated with age in cows [$P<0.001$, 6].

Percentage of cows with higher level of IPA / IgG and IgA / ELISA titre in BS was more in G-III and G-II as compared to other groups (Figure 1a). But percentage of cows with higher level of IPA / IgG and IgA / ELISA titre in CM was more in G-I, G-II, G-III and G-IV, respectively (Figure 1b). It indicated an increase in proportion of cows with higher serum-IgG and cervical mucus-ELISA-titre with age. Fayemi [12] were also of the opinion that the proportion of Zebu cattle with sperm antibodies increased significantly with age ($P < 0.001$).

3.2. ASA in blood serum and cervical mucus of cows according to parity (n=40)

ASA, detected by IPA / ELISA titre in BS increased non-insignificantly ($p < 0.05$) / significantly ($p < 0.05$) from G-I to G-III and IgG/IgA were non-significantly higher ($p < 0.05$) in G-II (Table 2). Zraly et al., [7] detected higher concentrations of ASA in 537 pluriparus cows by ELISA. Waziri and Fayemi [6] postulated that the presence of circulating sperm antibody was significantly associated with parity ($P < 0.001$). There was no difference in IPA-ASA and IgG type antibodies in cervical mucus of cows with respect to parity number. A decrease in IgA in CM from G-I to G-III and again an increase in G-IV was observed. ELISA titre in CM was also non-significantly maximum ($p < 0.05$) in cows of G-III.

Table 1: *Presence of ASA (evaluated by IPA, Sperm Mar test and ELISA, Mean ± SE) in blood serum and cervical mucus of cross-bred cattle according to age*

G. N0./ Age (Years)	IPA (%)		SpermMar Test				ELISA titre (%)	
			IgG (%)		IgA (%)			
	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus
I/ <3 (n=7)	39.3±6.1[a] (19.7-66.2)	47.9±2.6[a] (43.4-52.6)	36.5±2.4[a] (30.1-46.8)	38.5±3.1[a] (32.5-42.5)	32.9±2.2[a] (26.3-42.6)	50.5±5.3[a] (40.8-59.1)	1228±529[a] (100-3200)	226±108[a] (50-800)
II/ 3-4.5 (n=16)	53.1±3.4[a] (29.3-65.5)	40.6±4.4[a] (29.6-65.5)	43.0±2.6[b] (29.6-65)	44.6±2.5[a] (31.1-57.8)	36.3±3.7[a] (18.7-63.7)	41.7±3.6[a] (18.6-65.7)	2675±1181[b] (400-6400)	290±183[a] (25-800)
III/ 4.6-6.0 (n=7)	45.7±5.4[a] (28.9-64.7)	34.8±6.3[a] (14.3-58.1)	26.7±6.6[c] (17.6-57.7)	40.5±4.5[a] (27.4-56.8)	38.5±5.3[a] (19.8-52.1)	46.1±6.8[a] (20.6-64.1)	2971±657[c] (1600-6400)	253±105[a] (25-800)
IV/>6.0 (n=9)	43.7±5.3[a] (13.5-63.7)	33.3±6.3[a] (16.9-64.2)	35.5±5.8[c] (23.3-63.5)	45.1±7.9[a] (24.1-88.1)	31.6±6.3[a] (13.2-65.8)	39.1±8.4[a] (17-78.7)	3555±1273[c] (1600-12800)	300±78[a] (100-800)

- Figures in parentheses represent range of values
- Values with different superscripts are significant ($p<0.05$)

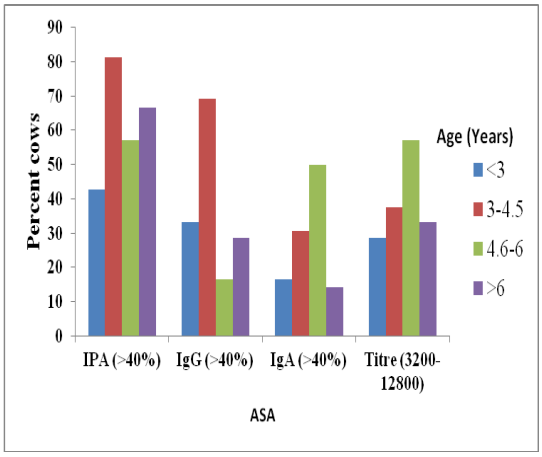

Figure 1a: *Occurrence of significant level of ASA in blood serum of cows according to age*

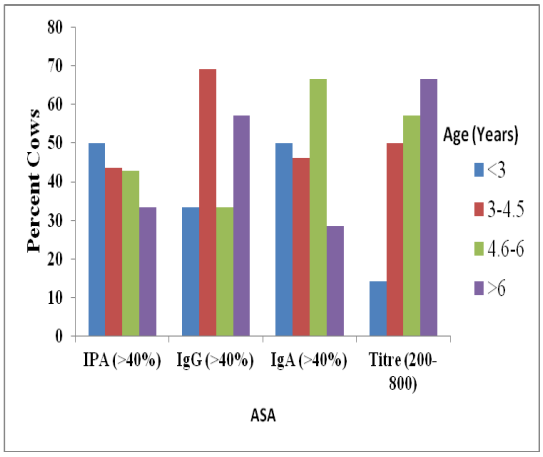

Figure 1b: *Occurrence of significant level of ASA in cervical mucus of cows according to age*

Percentage of cows with higher level of ASA detected by IPA and ELISA-titre in BS was higher in G-III and that of IgG and IgA in G-II and G-III as compared to other groups (Figure 2a). Percentage of cows with higher level of IPA / IgA and IgG / ELISA titre in CM was maximum in G-I and G-III, respectively (Figure 2b). It revealed that proportion of cows with higher ELISA titre in BS and CM increased with increased parity. The proportion of Zebu cows positive for sperm antibodies was significantly associated with increased parity [$P < 0.00$, 7]. Parturition can induce injury to the reproductive tract especially in cases of dystocia and injury to the reproductive tract may play a role in

induction of immunity against sperm [15]. This can be the reason for higher percentage of cows with significant level of ASA in G-I and G-III, as compared to G-II and G-IV.

Table 2: *Presence of ASA (evaluated by IPA, SpermMar test and ELISA, Mean ± SE) in blood serum and cervical mucus of cross-bred cattle according to parity*

Parity No (G. No.)	IPA (%)		SpermMar Test				ELISA titre (%)	
			IgG (%)		IgA (%)			
	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus
0 (G-I) N=7	39±5.6[a] (19.7-62.8)	40.1±10[a] (31.6-59.8)	38±2.8[a] (28.2-49.7)	41.5±9.6[a] (17.8-63.2)	35.4±4.2[a] (27.2-58.2)	49.9±9.2[a] (25-69)	1834±881[a] (100-6400)	200±70[a] (20-200)
1 (G-II) N=9	49.5±5.8[a] (29.3-79.5)	37.7±6.8[a] (17.6-63.5)	44.4±5.9[a] (31.6-65)	41.2±5.0[a] (24.1-57.8)	39.1±5.4[a] (18.7-58.5)	43.9±3.6[a] (30.9-57.5)	2750±798[b] (400-6400)	195±91[a] (40-800)
2 (G-III) N=16	52.1±3.2[a] (28.9-67.4)	37.9±5[a] (11.4-63.5)	37.8±3.9[a] (15.7-57.7)	43.9±5.0[a] (16.4-88.1)	30.7±2.1[a] (19.8-49.2)	37.5±3.6[a] (18.6-57.6)	3437±820[b] (400-12800)	335±65.9[a] (20-800)
≥3 (G-IV) N=9	44.4±5.5[a] (13.5-67)	34.3±6.3[a] (16.9-72.8)	31.1±3.8[a] (17.6-48.6)	41.8±7.1[a] (25.6-88.1)	37.3±6.1[a] (13.2-65.8)	42.7±7.6[a] (17-78.7)	2933±1250[b] (800-12800)	228±79[a] (50-800)

- Figures in parentheses represent range of values
- Values with different superscripts are significant (p<0.05)

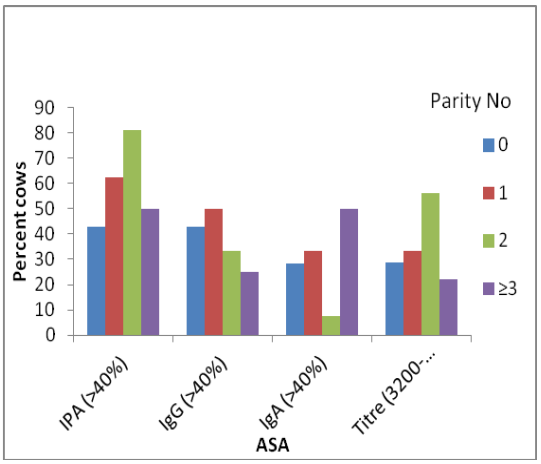

Figure 2a: *Occurrence of significant level of ASA in blood serum of cows according to parity number*

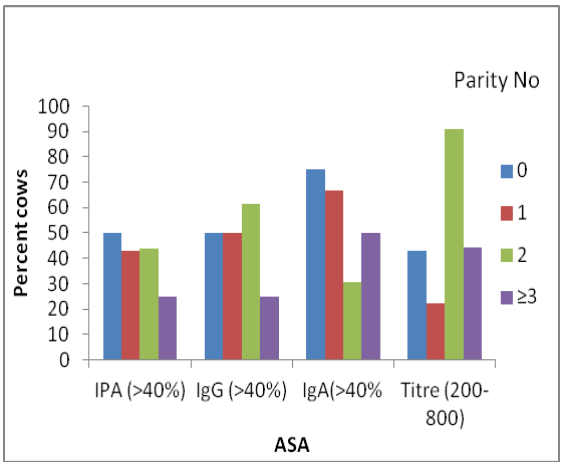

Figure 2b: *Occurrence of significant level of ASA in cervical mucus of cows according to parity number*

3.3. ASA in blood serum & cervical mucus of cows according to num. of inseminations (n=53)

ASA, detected by IPA showed a significant (p<0.05) increase in blood serum of cows from GI to GIII (Table 3). There was not any significant (p<0.05) difference in IgG in serum among the groups. However, IgA class antibodies and ASA titre in blood serum of cows showed an increase with increase in number of inseminations i.e. G-I to G-III. Results of Lazarevic et al. [16] indicated that titers of antisperm antibodies of the IgA class elevated with the number of artificial inseminations. Sarna et al. [11] observed the presence of ASA in serum of 100% cows repeating 3-5 times with a titre of 1:3120. It has been proposed that ASA positive rate varies according to the mating number and reproductive status. In the sera and cervical mucus of cows, high levels of ASA were found in animals with longer open day's period [17].

Table 3: *Presence of ASA (evaluated by IPA, SpermMar test and ELISA, Mean ± SE) in blood serum and cervical mucus of cross-bred cattle according to number of inseminations*

Number of AI (G. No.)	IPA (%)		SpermMar Test				ELISA titre (%)	
			IgG (%)		IgA (%)			
	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus
0 (G-I) N=3	27±3.7[a] (19.7-31.1)		35.4±3.1[a] (30.1-40.8)		31.5±0.4[a] (30.8-32.2)		333±233a (100-800)	
1-3 (G-II) N=25	45.4±2.9[b] (13.5-70.3)	32.6±3.1[a] (11.4-70.4)	34.8±2.6[a] (17.6-57.5)	36.6±2.7[a] (13-57.8)	38.3±3.5[b] (13.2-63.7)	43.1±3.7[a] (17-68.4)	1616±184[b] (400-3200)	205±45[a] (25-800)
4-6 (G-III) N=18	51.6±3.3[b] (28.9-79.5)	43.8±16.7[a] (28-72.8)	37.8±4.3[a] (15.7-65.6)	48.4±4.9[a] (27.1-88.1)	44.6±3.2[c] (19.4-55.8)	47±3.5[a] (29.1-69.4)	4222±876[c] (800-12800)	279±68[a] (25-800)
>6 (G-IV) N=7	49.2±6.5[b] (23.9-68.1)	34.8±6.1[b] (17.9-65.1)	38±6.9[a] (13.9-57.7)	37.6±5.8[a] (17.9-57.4)	40.1±7.2[c] (17.8-73.8)	42±3.3[a] (30.9-49.4)	4114±1501[c] (1600-12800)	133±26[a] (100-200)

- Figures in parentheses represent range of values
- Values with different superscripts are significant ($p<0.05$)

Percentage of cows with significant level of IPA, IgG and ASA titre in BS was maximum in G-IV, whereas that with IgA in G-III in comparison to other groups (Figure 3a). Percentage of cows with significant level of IPA, IgG, IgA and ELISA-titre were higher in G-III as compared to G-I, G-II and G-IV (Figure 3b). Tripathi et al. [9] revealed variable occurrence of ASA in repeat breeders. In our study percentage of cows that repeated 4-6 times were higher with significant level of ASA. Wang and Xie [8] studied ASA by ELISA in Chinese black and white dairy cows and found that prevalence of ASA was greatest (17.65%) amongst the cows that had three or more inseminations.

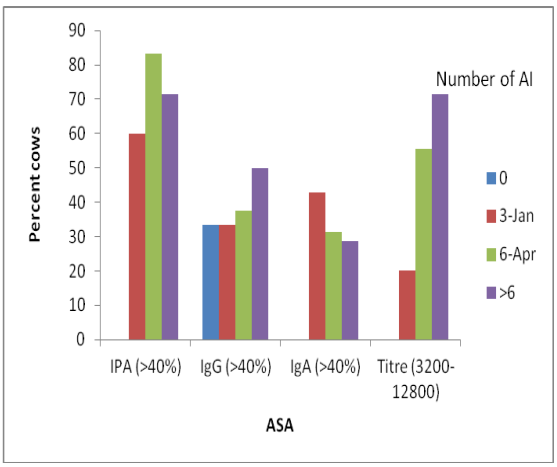

Figure 3a: *Occurrence of significant level of ASA in blood serum of cows according to number of inseminations*

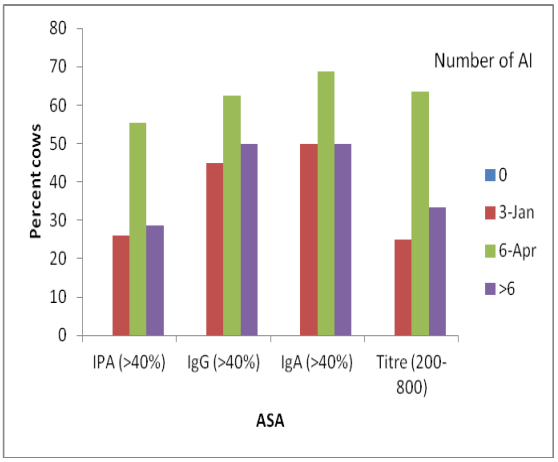

Figure 3b: *Occurrence of significant level of ASA in cervical mucus of cows according to number of inseminations*

Milovanović et al. [18] stated that the critical titre value of the antisperm IgA from CM in cows is 1:64, as of AI in the last oestrus and number of AI per pregnancy were significantly higher in cows that had IgA - ASA titer above this value. During the present study, ELISA titre in CM first showed an increase upto 3 - 4.5 years of age, second parity and 4-6 inseminations and then a decrease after 4.5 years of age, second parity and > 6 inseminations. During the period of estrus CM contains different amounts of water according to the hormonal status and therefore samples can be diluted several times. Consequently the titers of ASA will be lower but since this phenomenon is an individual characteristic,

the degree of dilution will not be the same in all animals. This is probably one of the causes for the high individual variability and along with the individual immune response, this result in large standard deviations and the lack of statistical significance for the obtained differences.

4. Conclusions

It can be concluded from our study that ASA were present in blood serum and cervical mucus of cross-bred cows irrespective of age, parity and number of inseminations. A significant increase in ASA in serum/cervical mucus was observed with increase in number of inseminations, but no significant difference was observed according to age and parity.

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Predominant Prevalence of *Brucella abortus* biovar-4 in Small Ruminants in Tamilnadu

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Abstract Brucellosis being a global zoonotic disease and animal health problem most of the studies has been oriented towards bovine than small ruminants. Hence the present study is conducted to identify the biotype of predominant *Brucella spp*. in small ruminants for defined control and eradication strategies. The study was carried out by random collection of the vaginal swab, milk, blood samples from the 315 sheep and goat. The samples were subjected for isolation and identification by biochemical tests followed by bio-typing with mono-specific A and M anti-sera and confirmed by multiplex AMOS PCR. Out of 195 goats and 120 sheep, 20 (10.25%) goats and 22 (18.33%) sheep were positive by culture with overall prevalence 13.33%. Fifteen isolates from goats and 20 isolates from sheep were typed as *B. abortus biovars*-4, 3 isolates from goats were typed as *B. melitensis* biovars-2, and 2 isolates from goats were positive for *B. ovis* and also 2 isolates from sheep were un-typed biovars. The confirmation was carried out by using multiplex AMOS-PCR. The study revealed that the *B. abortus* biovar-4 was predominant in small ruminants. Since most of the control strategies are directed towards bovine brucellosis this study indicates that small ruminant brucellosis control strategy must be redefined since it is the potential source of zoonosis.
Keywords *Brucellosis; Zoonotic; AMOS PCR; Brucella abortus biovar-4*

1. Introduction

Brucellosis is an infectious disease of domestic and wild animals with serious zoonotic implication in humans. Animal brucellosis is mainly characterized by reproductive involvement resulting in abortion and infertility whereas human brucellosis leads to chronic debilitating complications. Currently, the genus consist of 10 species classified based on their host preference and phenotypic differences. *B. abortus, B. melitensis* and *B. suis* are divided into 7, 3 and 5 biovars. Ovine and caprine brucellosis caused by *Brucella melitensis*, sporadic infection in sheep and goats can also be caused by *Brucella abortus, Brucella ovis* or *Brucella suis* (OIE, 2009). Clinical disease is common in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and Caribbean. In India, brucellosis was first recognized in 1942 and is now endemic throughout the country. *B. abortus*

biotype-1 in cattle and buffaloes and *B. melitensis* biotype-1 in sheep, goats and man are the predominant infective biotypes in India (Renukaradhya et al., 2002). The disease is mainly manifested as reproductive failure which includes abortion in pregnant female, stillbirth, placentitis, epididymitis and orchitis in male. Localization may also occur in mammary tissue with excretion in the milk (Pal, 2007).

The present study has been undertaken to estimate the prevalence of brucellosis in sheep and goats by culture and biovar identification by biochemical tests and multiplex AMOS-PCR assay. This study of biovar identification will be helpful for effective vaccine preparation for the control of brucellosis in small ruminants.

2. Materials and Methods

The samples were collected from 120 sheep and 195 goats in Tamilnadu. A total of 80 milk samples (38 from sheep and 42 from goats), 299 vaginal swabs (120 from sheep and 179 from goats), 210 whole blood (120 from sheep and 90 from goats) and 16 aborted materials samples from goats were collected from organised and unorganised sheep and goat units.

2.1. Isolation and Identification

Isolation from single animal is a sufficient evidence to establish the infection status of a herd and is considered to be the Gold standard test (OIE, 2009; Kaltungo et al., 2014). The milk, vaginal swab and aborted materials were cultured in *Brucella* selective media (Himedia) containing *Brucella* selective supplement (Himedia). The organism were isolated from whole blood by lysis centrifugation technique as per mentioned by Mantur (2004). Identification of *Brucella* organism were carried out by Grams staining, modified acid fast staining followed by bio-typing with biochemical tests *viz*., oxidase, catalase, urease, H_2S production, growth in the presence of thionin and basic fuchsin dye and agglutination with mono-specific A and M antisera as described by Alton et al. (1988).

2.2. Polymerase Chain Reaction

The isolates were grown on *Brucella* selective media and incubated for 48 hours at 37°C. The DNA were isolated from pure culture by using one step medox DNA extraction reagent. Few colonies from culture were suspended in 100 µl of medox reagent in ependorf tubes and incubated at 100°C for 15 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes and the supernatant containing crude DNA were collected into sterile ependorf tubes. The DNA was stored at -20°C until use.

AMOS-PCR (*B. abortus, B. melitensis, B. ovis* and *B. suis* – Polymerase Chain Reaction) was carried out as described by Bricker and Halling with minor modification (Bricker and Halling, 1994). A total of 25µl PCR assay reaction mixture consisted of 2x PCR mastermix (Apliquon), four sets of primer (20 pmol each) *B. abortus, B. melitensis, B. ovis* and IS711 -specific primer (Table 1) and 5 µl DNA template.

Table 1: *Primer sequences* (Bricker and Halling, 1994)

Name of Primers	Sequences (5´-3´)
Brucella abortus specific	GAC GAA CGG AAA TTT TCC AAT CCC
Brucella melitensis specific	AAA TCG CGT CCT TGC TGG TCT GA
Brucella ovis specific	CGG GTT CTG GCA CCA TCG TCG
IS711 specific	TGC CGA TCA CTT AAG GGC CTT CAT

The PCR was performed for 35 cycles in thermocyclar, each cycle consist of initial denaturation at 94°C for 1 minute, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extention at 72°C for 1 minute. The final extention of PCR product was carried out at 72°C for 10 minutes. The PCR products were separated by 1.5 % agarose gel electrophoresis at 100 V for 45 minute and gel documented. AMOS-PCR shows amplicon of size 498 bp for *B. abortus*, 731 bp for *B. melitensis* and 976 bp for *B. ovis*.

3. Results

A total of 45 isolates, 20 (10.25%) from goats and 22 (18.33%) from sheep were identified as gram negative coccobacilli and positive for modified acid fast staining. Biotyping was carried out by biochemical tests *viz.,* oxidase, catalase, urease, H_2S production, growth in the presence of thionin and basic fuchsin dye and agglutination with monospecific A and M antisera. Fifteen isolates from goats and 20 isolates from sheep were typed as *B. abortus* biovar- 4, 3 isolates from goats were typed as *B. melitensis* biovars- 2, and 2 isolates from goats were positive for *B. ovis* and also 2 isolates from sheep were un-typed biovar (Table 2). All 42 isolates were detected by the AMOS-PCR and 35 isolates confirmed as *B. abortus* (498 bp), 3 isolates belong to *B. melitensis* (731 bp), 2 isolates identified as *B. ovis* (976 bp) and 2 isolates identified as un-typed *B. abortus* biovar (498 bp) and 3 isolates were showed negative to PCR. (Figure 1)

Table 2: *Result of biochemical test for Brucella Isolates*

Species	Total no. of isolates	Oxidase	Catalase	Urease	H_2S Production	Growth in the presence of Dye		Agglutination with Monospecific antisera		Biovar typing
						Thionin	Fuchsin	A	M	
Sheep	20	+	+	+	+	-	+	-	+	***Brucella abortus*** **biovars -4**
Goat	15	+	+	+	+	-	+	-	+	
Goat	3	+	+	+	-	-	+	+	-	***Brucella melitensis*** **biovar -2**
Goat	2	+	+	-	+	+	-	-	-	***Brucella ovis***
Sheep	2	+	+	+	+	+	+	+	+	**Untyped biovar**

Thionin : 20µg/ml (1:25,000) +: positive
Basic fuchsin dye : 20µg/ml (1:25,000) - : negative

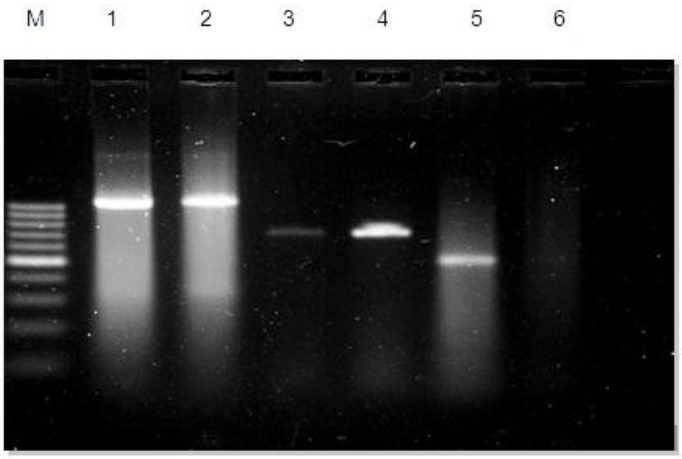

Figure 1: *AMOS-PCR*

Lane M: 100 bp ladder; Lane 1-2: *B. ovis* (976 bp); Lane 3-4: *B. melitensis* (731 bp); Lane 5: *B. abortus* (498 bp); Lane 6: Negative Control

4. Discussion

Brucellosis is a communicable disease endangering the efforts to improve productivity in sheep and goat. Determining the biovar is an important step for epidemiologic characterization of the disease and also provides preliminary data to design control strategies with regard to vaccine development. Though eradication of brucellosis in small ruminants can be achieved by depopulation of infected flocks, in developing country like India, this strategy is very difficult to adopt. Further, extensive type of rearing, nomadic system, common grazing ground and transhumance of the flock makes it difficult to design a suitable control strategy. Since brucellosis is endemic, the first step is directed to control the susceptible flock by immunization with appropriate vaccine strain. In this regard this study was carried out to identify the most prevalent brucella biovar among small ruminants.

Out of 315 samples collected from sheep and goat, 42 isolates were recovered from milk, vaginal swab, blood and aborted material. Percentage of recovery from milk, vaginal swab, aborted material and whole blood were 11.90%, 21.42%, 11.90% and 54.76% respectively. As brucella is intracellular organism percentage of recovery found to be more from whole blood samples.

Brucellosis is a worldwide zoonotic disease that is recognized as a major cause of heavy economic losses to the livestock industry and poses serious human health hazard. It is considered to be the most important cause of abortion and reduced fertility in infected sheep and goats. Determination of biovars is an important step for epidemiologic characterization of the disease and provides preliminary requirements for designing of control and eradication programs. The present study was undertaken for identification of *Brucella* biovars from small ruminants based on the biochemical tests and confirmation and differentiation of *Brucella* spp. was carried out by multiplex AMOS PCR.

Based on isolation the prevalence among sheep and goat were recorded as 18.33% (22 no.) and 10.255 (20 no.) respectively. Following isolation the isolates were conventionally characterized by biochemical tests viz, oxidase, catalase, urease, H_2S production, growth in presence of thionin and fuchsine dye and agglutination with monospecific sera A and M. Di Giannatale et al. (2008), Affi et al. (2011), Behroozikhah et al. (2012) was used these tests for characterization of brucella biovars. Isolates were further confirmed by multiplex AMOS PCR was carried out for confirmation and differentiation of *Brucella* species and the result recorded. Among 42 isolates 39 isolates were positive and 3 isolates found negative which could be due to different strain of *Brucella* organism. Ancora et al. (2005) and Matope et al. (2009) used biochemical profile and AMOS–PCR for the differentiation and typing of *B. abortus* and *B. melitensis* biovar. Similarly Mirnejad et al. (2013) recorded 45.3% *B. abortus* and 54.6% *B. melitensis* from culture positive samples by multiplex PCR.

In the present study, it was found that out of 42 isolates 35 were identified as *B. abortus* biovar-4 (83.33%), 3 isolates as *B. melitensis* biovar-2, 2 isolate identified as *B. ovis* and 2 isolates were typed as un-typed biovar. In 1975 Sen and Sharma recorded *B. abortus* biovar 4, 6, and 9 from cattle and buffalo whereas *B. melitensis* biovar 2 from sheep and goats. On contrary to this majority of the isolates were identified as *B. abortus* biovar-4 among small ruminants. The possible reason could be in organized farms sheep and goats are reared alongside dairy units and transmission could be through person, vehicle, fodder, drainage etc. In un-organized farm, since sampling has been done from small farmers they share a community pastures land which could be the source of infection. It needs to be assessed whether *B. abortus* biovar 4 has higher transmission ability than other biovars. Further work need to be carried out to find the ability of *B.abortus* biovar-4 to produce either abortion or clinical disease in small ruminants or to act as carriers in spreading the disease.

5. Conclusion

The findings of the study revealed higher distribution of *B. abortus* biovar -4 among the small ruminants which found mainly in cattle and buffalo as reported by Sen and Sharma (1975). The presence of *B. abortus* biovar -4 in sheep and goats might be due to integrated farming system comprising of infected and healthy cattle which shares a common pasture land. The untyped biovar could be due to emerging of new strain of *Brucella* in small ruminants. Vaccine developed with the prevalent strain may help to reduce the disease in both cattle and small ruminants.

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