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Molecular characterization of *Staphylococcus* species isolates from buck semen and their effect on semen quality

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ABSTRACT

Objective: Buck reproductive health is the key for breeding and production of quality semen. To assess the health of breeding bucks, in this study, we detected the presence of *Staphylococcus* spp in semen. *Staphylococcus aureus* is a common commensal and opportunistic pathogenic bacteria and is also a cause of many diseases in animals. Besides this, it can also deteriorate the semen quality.

Materials and Methods: In this study, we collected 48 semen ejaculates from healthy bucks of three breeds, namely, Jamunapari, Barbari, and Jakhrana to assess the presence of *Staphylococcus* spp. Besides bacteriological study, the semen was also assessed for semen quality parameters in infected as well as in non-infected semen samples.

Results and Conclusion: The semen quality was significantly deteriorated with *Staphylococcus* infection. The bacterial infection was initially confirmed as *Staphylococcus* spp. based on the Gram's staining and growth on Mannitol salt agar. Based on this preliminary bacteriological analysis, 52.08% (n = 25) of the samples were found positive for *Staphylococcus* spp. from the total 48 buck semen ejaculates belonging to three different goat breeds. The isolates were confirmed based on the basis of multiplex PCR and the species identified were *S. aureus*, *Staphylococcus sciuri, Staphylococcus haemolyticus, Staphylococcus chromogenes, Staphylococcus epidermidis,* and *Staphylococcus simulans* directly in the buck semen. By this study, it is evident that semen can get contamination from a buck which has the presence of staphylococcus in the reproductive tract and semen quality is adversely affected. Hence, it is important to ensure the health and hygiene of the bucks maintained for semen production for artificial insemination.

Keywords: Buck semen, Goat breeds, Buck health, *Staphylococcus* spp

INTRODUCTION

In the present scenario, goat artificial insemination (AI) is gaining importance, but the success of AI in goats is dependent on the efficient semen cryopreservation techniques. Bacterial contamination can compromise the quality of buck semen due to its spermicidal effects. Bacteria are present in every semen ejaculate; most of them being commensal bacteria, but some of them may be harmful pathogens. However, by following proper precautions, ensuring healthy and hygienic maintenance of breeding bucks can minimize the contamination and hence will aid in production of quality semen.[1,2] *Staphylococcus aureus* is the most common Gram-positive bacteria and it is also involved in variety of infections.[3] Various virulence factors include protein A, Panton Valentine Leukocidin, coagulases, certain toxins such as surface and pore

forming toxins, superantigens, and entertoxins that aids in establishing successful disease conditions in host.[4]

Male genital tract is exposed to the environment and use of artificial vagina (AV) and other semen collection tools could act as fomites in spreading the infection. However, use of quality tools with proper hygiene and maintenance of animals in clean environment could minimize the affections of pathogens. Hence, a mechanism is required to screen the bucks for presence of pathogens and its regular screening. In the present study, we have bacteriologically tested the buck semen for the presence of various staphylococcal species that can cause possible affections in the semen quality and in turn be a threat to the female fertility and conception rate. Infections of the male genitourinary tract lead to infertility by affecting spermatogenesis, inflammations of male reproductive tract, obstruction of epididymis, and stress leading to poor quality semen.[5]

These pathogens are also the important carrier of antimicrobial resistance factors such as Methicillin-resistant *S. aureus* and semen contaminated with such microbes can compromise the safety of personnel involved in the AI. Moreover, these pathogens or other attributes can be a direct threat to the breeding does inseminated through AI. Hence, the experiment was planned to investigate the presence of *Staphylococcus* isolates from the semen samples of bucks along with their molecular speciation using multiplex Polymerase Chain Reaction (PCR) and their effect on semen quality.

MATERIAL AND METHODS

Semen collection

Semen ejaculates (n = 48) were collected aseptically following the standard conditions using AV from bucks of breeds, namely, Barbari (n = 16), Jamunapari (n = 16), and Jakhrana (n = 16) with similar age group. Four bucks were maintained for each breed and four ejaculates were collected per buck on different days of the breeding season. Semen samples were collected applying separate AV in each collection that was sterilized by autoclaving and following hygienic methods as described previously.[6] Besides, all the bucks belonged to same breeding season and maintained in similar housing and microenvironmental conditions.

Semen evaluation

Mass motility of the sperms in fresh semen was estimated at low power magnification (×10) using a compound microscope on microscopic thermostage maintained at 37°C. Semen samples were diluted at 400 million spermatozoa per milliliter, and the diluted semen (10 µl) was put on a clean pre-warmed slide (37°C) with cover slip and observed under ×40 objective of phase contrast microscope for observing the progressive motility. Sperm viability was evaluated following the procedure mentioned.[7] Sperm abnormalities were counted according to the method described.[5] Giemsa stain was taken to evaluate the acrosomal integrity of buck spermatozoa as per the method described.[8] The hypo-osmotic swelling test was done to evaluate the functional integrity of the sperm plasma membrane. Plasma membrane integrity was examined as per the protocol used.[9]

Microbiological evaluation of semen

Semen samples collected were immediately inoculated into nutrient broth and the growth obtained after incubation at 37°C for 16 h was then sub-cultured into nutrient agar and mannitol salt agar as per the previously described method.[10] The growth from the broth culture/solid media was confirmed by Gram's stained smears and was identified based on the morphology at ×100 objective of the microscopy under oil immersion.[11]

Molecular characterization of *Staphylococcus* spp.

Isolates of *Staphylococcus* spp. obtained were further isolated for its DNA using freeze-thaw method. This method was developed in the present study for easy extraction of PCR-grade quality DNA. In this method, an overnight broth culture or a loopful suspension of culture from an isolated colony was centrifuged at 8000 rpm at room temperature for 10 min. The clear supernatant was decanted gently while preserving the bacterial pellet and it was reconstituted with sterile PBS (PH 7.2) and washed for 3 times. The bacterial pellet was further resuspended in DNase free water and frozen at −40°C for 1 h followed by boiling at 100°C for 2 min. The cycle was repeated thrice. The mixture was finally centrifuged and collected the supernatant containing the soluble DNA. The DNA was quantified using Quantus™ fluorometer as per the protocol given by the manufacturer. The DNA thus quantified was used as a template for diagnostic PCR[12] with certain modifications and conditions as described.[2]

Statistical analysis

Data are presented as mean ± SE and the statistical analysis was done by SPSS Software version 22.0, IBM (SPSS Inc., Chicago, IL, USA). The differences in mean values were estimated using paired t-test. The semen ejaculates were considered as experimental units. The difference between means was significant at 95% level of significance ($P < 0.05$).

RESULTS

Isolation and identification of *Staphylococcus* from buck semen

The *Staphylococcus* isolates thus obtained were based on the Gram's staining, growth on mannitol salt agar and catalase test

is tabulated [Table 1 and Figure 1]. In this study, we could isolate 25 (52.08%) *Staphylococcus* spp. isolates from semen samples.

Semen evaluation parameters

All the collected semen samples were evaluated for mass motility, progressive motility, viability, acrosomal integrity, and plasma membrane integrity. The semen samples positive for microbial contamination showed significant ($P < 0.05$) decrease in sperm motility, acrosomal integrity, and plasma membrane integrity, as well as significant ($P < 0.05$) increase in sperm abnormalities Table 2.

Molecular characterization of *Staphylococcus* Spp.

After initial identification of bacteria as *Staphylococcus* spp., further confirmation of bacteria was done on the basis of molecular characterization using multiplex PCR as described elsewhere.[2] Genotypically, the species identified were *S. aureus*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus Simulans*, and *Staphylococcus epidermidis* in the buck semen as given in [Table 3].

Table 1: Incidence of Staphylococcosis in buck semen.

Breed	Total no. of semen ejaculates tested	Semen ejaculates positive for *Staphylococcus no.* (%)
Barbari	16	7 (43.75)
Jamunapari	16	8 (50.00)
Jakhrana	16	10 (62.50)
Total	48	25 (52.08)

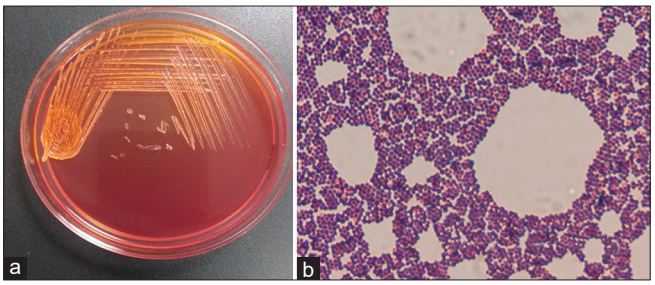

Figure 1: (a) *Staphylococcus aureus* with lemon yellow colonies on mannitol salt agar showing fermentation of mannitol with characteristic yellow zone around the colonies of. (b) *S. aureus* appearing as Gram-positive cocci arranged as characteristic "bunch of grapes."

DISCUSSION

This study was primarily designed to corroborate the phenotypical parameters of semen vis-à-vis the presence of *Staphylococcus* spp. in the semen from selected bucks maintained in healthy environment. Furthermore, the effect of *Staphylococcus* on semen quality was assessed. Various species of *Staphylococcus* were detected from breeding buck's semen by multiplex PCR. About 52% of the semen samples obtained from different breeds of goats were found positive for *Staphylococcus* species which was confirmed by Gram's staining as well as growth on mannitol salt agar. In Barbari – 43.75%, Jamunapari – 50%, and Jakhrana – 62.50% were found to harbor various species of *Staphylococcus* in its semen [Table 1]. A similar pattern was also observed in the earlier study in bucks.[2]

The presence of *Staphylococcus* in buck semen has a direct effect on the semen, as we found significant reduction in the progressive motility, acrosomal integrity, and plasma membrane integrity and significant increase in sperm abnormalities [Table 2]. In earlier studies, it is reported that *S. aureus* can easily invade the reproductive tract affecting its functions[6,13] and reducing the reproductive potential of spermatozoa.[14] Most studies conducted in the past also reported that *S. aureus* and *S. epidermidis* are the most common *Staphylococcus* spp. reported in bull semen and the metabolic by-products secreted from these species were reported to have damaging effects on the acrosomal integrity and sperm motility.[15] Similarly, a study conducted on ram semen ejaculates reported the presence of *S. aureus* and *S. epidermidis* and other Gram-negative bacteria belonging to enterobacteriaceae were found in higher proportion of semen samples.[16] Similar studies were conducted in buffalo, which too reported *Staphylococcus* species in its semen.[17] *S. aureus* affects the sperm function by hampering the metabolic activity of spermatozoa and also reducing its viability. We found that the prevalence of *S. aureus* strains [Table 1] was 52.08 %, which was similar to the previous reports.[18] In the present study, we could confirm various species of *Staphylococcus* including *S. aureus*, *S. chromogenes*, *S. haemolyticus*, *S. sciuri*, *S. simulans*, and *S. epidermidis* in the buck semen [Table 3]. The isolation of these organisms and its effect on sperm morphology and function has been poorly investigated globally. Earlier studies reported that aerobic cocci are present in about 50% of semen samples of male partners having fertility related issues.[19] Diemer *et al*. (2003) reported that the most commonly isolated bacteria from male animals with urogenital tract infections or semen contamination are *Escherichia coli*.[20] However, Enwurua *et al*. (2016) reported that *Staphylococcus* spp. is the most predominant microorganism of urogenital infections.[21] In the present study, besides *S. aureus*, we have obtained considerable number of isolates of other species including *S. chromogenes*, *S. hemolyticus*, *S. simulans,* and *S. sciuri* by molecular detection.

In the present study, there is significant decrease in progressive motility, acrosomal integrity, and plasma membrane integrity in bacterial contaminated semen

Table 2: Semen quality: Positive versus negative for the presence of *Staphylococcus*.

S. No	Parameters	Semen samples positive for *Staphylococcus*	Semen samples negative for *Staphylococcus*
1.	Mass motility (0–5)	3.77±0.08	3.91±0.09
2.	Progressive motility (%)	75.67±1.05[b]	80.24±0.92[a]
3.	Sperm abnormalities (%)	12.56±0.17[a]	7.80±0.06[b]
4.	Sperm viability (%)	86.22±1.34	88.45±1.55
5.	Acrosomal integrity (%)	82.56±1.08[b]	88. 80±1.40[a]
6.	Plasma membrane integrity (%)	78.72±0.95[b]	82.84±1.02[a]

*Means (±SE or SEM) bearing different superscripts are statistically different. ([a]$P<0.05$; [b]$P<0.05$)

Table 3: Species of *Staphylococcus* spp. isolated and identified from buck semen based on multiplex PCR.

S. No.	Species identified	Type of genes (amplicon size)	Number (%)
1.	*Staphylococcus aureus*	*23SrRNA* (894bp), *Nuc* (278bp)	11 (44)
2.	*Staphylococcus chromogenes*	*Sod*A (222bp)	4 (16)
3.	*Staphylococcus haemolyticus*	*Sod*A (214bp)	2 (8)
4.	*Staphylococcus sciuri*	*Gap* (306bp)	4 (16)
5.	*Staphylococcus simulans*	*Gap* (472bp)	2 (8)
6.	*Staphylococcus epidermidis*	*Rdr* (130bp)	2 (8)
		Total isolates	25 (52.08)

[Table 2]. In concurrence to the present findings, earlier, Huwe *et al*. (1998) studied the effect of different pathogenic microorganisms on human sperm motility parameters and found that *S. aureus* negatively influences sperm motility.[17,22] In another study conducted by Liu *et al*. (2018) also reiterated the fact that semen quality is deteriorated while infected with *S. aureus*.[18] The declined spermatozoa motility may be due to the inhibition of ATPase pump of spermatozoa.[23] Earlier researchers have reported that bacterial interaction with spermatozoa hampers the sperm motility,[24-26] while some researchers have reported that bacteria in semen are responsible for the production of soluble spermicidal factor in the extracellular medium which is responsible for the reduced sperm viability and acrosomal damage.

CONCLUSION

The association of a specific bacteria and its effect in semen quality has been a topic of least importance especially in domestic animals. In this study, we tried to examine the effect of various *Staphylococcus* species on semen quality parameters. The phenotypical effect such as decrease sperm motility and the bacterial moieties can damage acrosomes and cause DNA damage by producing reactive oxygen species leading to poor conception rates and eventually affecting the financial returns**.**

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Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Conflicts of interest

There are no conflicts of interest.

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