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Comparative Efficacy of Antibiotics in Semen Dilutor on Bacterial Load and Sperm Quality in Cryopreserved Frieswal Bull Semen

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ABSTRACT

The study was conducted to evaluate the efficacy of the antibiotic combinations, streptopenicillin (SP) and gentamicin, tylosin, lincomycin and spectinomycin (GTLS) for the control of bacterial load and improvement of semen quality in Frieswal bulls (Holstein Fresian × Sahiwal). *Staphylococcus, Proteus, Klebsiella, Bacillus, Actinomyces, E. coli* were isolated from the frozen semen. The cultural sensitivity testing of the bacterial isolates showed that Gentamicin and Spectinomycin were most effective while penicillin was least effective against the isolated organisms. For the study, ejaculates from bulls ($n = 6$) were taken twice weekly for 3 weeks. Each ejaculate was divided into 3 aliquotes. Tris egg yolk citric acid buffer extender was used to dilute the semen samples. Streptopenicillin antibiotic combination was used in one aliquote while gentamicin, tylosin, lincomycin and spectinomycin were used in 2nd aliquote. In 3rd aliquote, no antibiotic was used and kept as untreated control. Semen straws were frozen from all three aliquots and semen quality parameters were analyzed as per standard procedures. Significantly better bacterial load control ($p < 0.05$) was observed in GTLS antibiotic combination added group as compared to SP group. Significantly ($p < 0.05$) higher post thaw sperm motility was recorded at 0 min, 30 min and 60 min after incubation at 37ºC in GTLS treated group as compared to SP treated group. Percentage of hypoosmotic swelling test and acrosome integrity were found significantly ($p <0.05$) higher in GTLS antibiotic group as compared to SP group. As regards to live percentage of spermatozoa, both SP and GTLS group had significantly higher live sperm concentration as compared to non-antibiotic group while they did not differ significantly with each other. Sperm abnormalities of head, mid piece and tail did not differ significantly within all the three groups. The study concludes that gentamicin, tylosin, lincomycin and spectinomycin combination is better than streptopenicillin in controlling bacterial load and improving semen quality.

HIGHLIGHTS

- Gentamicin, tylosin, lincomycin and spectinomycin (GTLS) antibiotic combination was effective in controlling bacterial load of bull semen.
- GTLS antibiotic combination was shown to improve sperm motility, sperm membrane integrity, and acrosomal intactness following cryopreservation.

Keywords: Antimicrobials, Bacterial load, Sensitivity, Semen quality

Bacterial contamination in semen can significantly impact sperm quality (Dela Pena *et al.,* 1995). Contaminants may originate from various parts of the bull reproductive tract viz. testes, epididymides, accessory sex- glands, vas deferens, and prepuce. Even with strict hygiene measures, contamination can occur during collection or processing, and cryopreservation carries a heightened risk for bacterial presence (Rana *et al.,* 2012; Sannat *et al.*, 2015). Bacteria impair semen quality directly by causing sperm agglutination, altering cell morphology, and reducing acrosome reaction capacity, or indirectly by generating reactive oxygen species due to inflammation (Moretti *et*

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al., 2009). These pathogens can be transmitted through natural mating or artificial insemination (AI), potentially decreasing female fertility (Andrabi *et al.,* 2016).

Since the preservation media for semen can promote microbial growth, antibiotics are commonly added to manage bacterial load. Typically, streptomycin and penicillin (SP) are used in bovine semen extenders; however, some bacteria have developed resistance to these antibiotics (Akhter *et al.,* 2008; Azawi and Ismaeel, 2012). Research suggests that an alternative antibiotic combination consisting gentamicin, tylosin, lincomycin, and spectinomycin (GTLS) may be more applicable, showing higher efficacy in culture tests against bacteria isolated from bull semen. This combination has also been shown to maintain semen quality post-thaw without adverse effects on fertility (Hasan *et al.,* 2001; Andrabi *et al.,* 2016). Therefore, the research was aimed to evaluate and compare the effectiveness of conventional SP combination and the newer GTLS combination in semen extenders, focusing on reducing bacterial load and enhancing semen quality in bulls.

MATERIALS AND METHODS

Experiment site and animals

The research was conducted at the Male Germplasm Unit of ICAR-Central Institute for Research on Cattle, located in Meerut, India. Six healthy and mature Frieswal (the first synthetic cattle breed of India) bulls aged between 3-6years were selected for this study. These bulls were individually housed in well-maintained pens designed with both covered and open areas, ensuring equal floor space per animal under a loose housing system that promotes animal welfare and comfort. To meet their nutritional requirements and maintain optimal health, the bulls were managed according to standard farm practices. They were provided unrestricted access to clean and fresh water and fed a carefully balanced diet. Each animal received approximately 22–25 kg of green fodder daily, supplemented with 6–7 kg of wheat straw. Additionally, a concentrate mixture containing 18% crude protein and 70% total digestible nutrients was provided at a rate of 3.5 kg per bull per day.

Determination of bacterial load in frozen semen

The bacterial colonies in frozen-thawed semen were assessed using the pour plate method. For this, a 0.1 mL aliquot of the frozen-thawed sample was added to a sterilized 5 mL glass tube containing 0.9 mL of PBS (pH 7.4) and serially diluted to 10-fold, 100-fold, and 1000-fold concentrations. For each dilution, duplicate sets of petri plates were used, and the bacterial load was determined following the method described earlier (Chand *et al.,* 2022). Bacterial identification was performed using Gram staining. Representative colonies were streaked onto selective media for further subculture and biochemical confirmation of the isolates, as per Holt *et al.* (1994).

Antibiotic Sensitivity Test

The in vitro antibiotic susceptibility of bacteria isolated from bull semen was assessed using the agar disc diffusion technique. Antibiotic discs containing specific concentrations—streptomycin (100 µg), penicillin G (10 units), gentamicin (10 µg), tylosin (15 µg), lincomycin (10 µg), and spectinomycin (100 µg) were employed following the standard procedure described by Quinn *et al.* (1994). After incubation, the diameter of the inhibition zones was measured in millimeters using a scale, and susceptibility or resistance was determined based on established reference values.

Semen collection, preparation of the extender and addition of antibiotics

Two ejaculates per week from six freezable Frieswal bulls were collected for 3 weeks and each ejaculate was divided into 3 aliquots (n=36). Tris egg yolk citric acid buffer extender was used for diluting the semen samples. Streptopenicillin conventional antibiotic combination was used in one aliquot (streptomycin @ 1 g per liter and Penicillin @ 1 lac IU per liter of extender) while gentamicin (@ 0.5 g per liter), tylosin (@ 0.1 g per litre), lincomycin (@ 0.3 g per liter) and Spectinomycin (@ 0.6 g per liter) were used in 2nd aliquot. No antibiotic was used in 3rd aliquot and kept as untreated control. Semen straws were frozen from all three aliquots.

Semen evaluation

To evaluate the sperm motility, 20 µL of frozen-thawed semen was positioned on a clean grease free glass slide and observed under light microscope (Olympus, Tokyo, Japan). Progressive motility (%) was assessed at 0, 30 and 60 minutes of incubation at 37°C. Sperm concentration was measured using Neubauer's chamber. For viability and sperm morphology Eosin-Nigrosine stain was used. Sperm membrane intactness was assessed by hypo-osmotic swelling test (HOST) and acrosome integrity was performed using Giemsa stain.

STATISTICAL ANALYSIS

The collected data were analyzed using SPSS version 20.0 for Windows (IBM SPSS® Statistics, USA). To assess differences in the mean values of various parameters across the experimental groups, Analysis of Variance (ANOVA) with Duncan's post-hoc test was employed as the primary statistical tool. Graphs were prepared using MS Excel and GraphPad Prism (version 5.0).

RESULTS AND DISCUSSION

The research was directed to assess the comparative efficacy of streptopenicillin (SP) and gentamicin, tylosin, lincomycin and spectinomycin (GTLS) antibiotic combinations for control of bacterial load and semen quality in Frieswal bulls. Bacterial organisms belonging to *Staphylococcus, Proteus, Klebsiella, Bacillus, Actinomyces, E. coli* were isolated from the frozen semen. The cultural sensitivity testing of the bacterial isolates showed that Gentamicin and Spectinomycin were most effective while penicillin was least operative against the isolated bacteria. Lincomycin, tylosin and streptomycin were moderately effective against the isolated bacterial organisms.

Better control of bacterial load (392.22 ± 58.97 cfu/ml) was observed in the group with GTLS antibiotic combination as compared to the streptopenicillin group (747.40 ± 107.82 cfu/ml Fig. 1). Significantly higher post thaw sperm motility was recorded at 0 min (52.63 ± 0.96), 30min (45.13 ± 0.83) and 60 min (36.25 ± 0.94) after incubation at 37ºC in GTLS treated group as compared to streptopenicillin treated group (0 min - 49.16 ± 0.42, 30 min-42.08 ± 0.67 and 60 min-33.75 ± 0.83 Fig. 2). Percentage of hypoosmotic swelling test (HOST- 47.76 ± 0.59) and acrosome integrity (73.34 ± 0.52) were found significantly ($p<0.05$) higher in GTLS antibiotic group in contrast to streptopenicillin group (HOST- 44.11 ± 0.69, acrosome integrity-70.32 ± 0.40 Fig. 3). As regards to live percentage of spermatozoa both SP (63.05 ± 1.01) and GTLS (65.30 ± 0.89) group had significantly higher live sperm concentration as compared to non-antibiotic group (control-57.24 ± 1.60) while they did not differ significantly with each other (Fig. 3). Sperm concentration did not differ significantly between the groups (Fig. 3). Sperm anomalies of head, middle piece and tail did not differ significantly amongst three groups (Fig. 4).

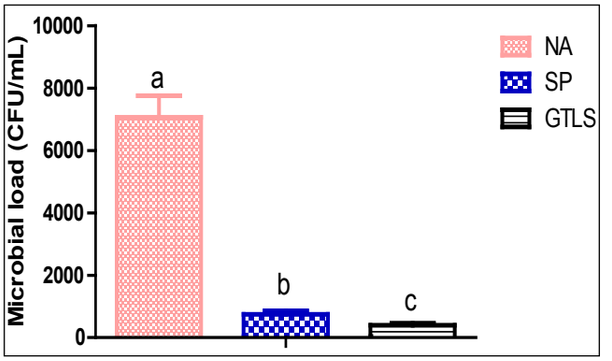

Fig. 1: Effect of antibiotics in semen extender on microbial load of breeding bulls. ($P<0.05$) NA- No antibiotic; SP- Streptopenicillin; GTLS- Gentamicin, Tylosin, Lincomycin, Spectinomycin

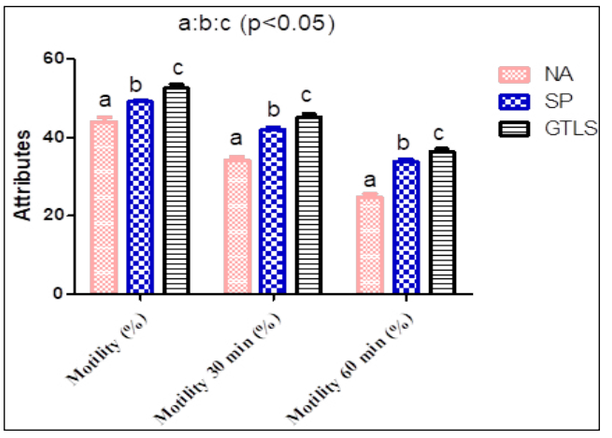

Fig. 2: Effect of antibiotics in semen extender on sperm motility of breeding bulls. NA- No antibiotic; SP- Streptopenicillin; GTLS- Gentamicin, Tylosin, Lincomycin, Spectinomycin

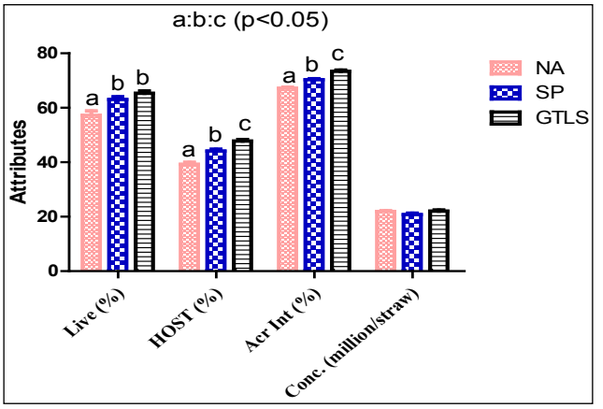

Fig. 3: Effect of antibiotics in semen extender on viability, HOST, Acrosome Integrity and sperm concentration of breeding bulls. NA- No antibiotic; SP- Streptopenicillin; GTLS- Gentamicin, Tylosin, Lincomycin, Spectinomycin

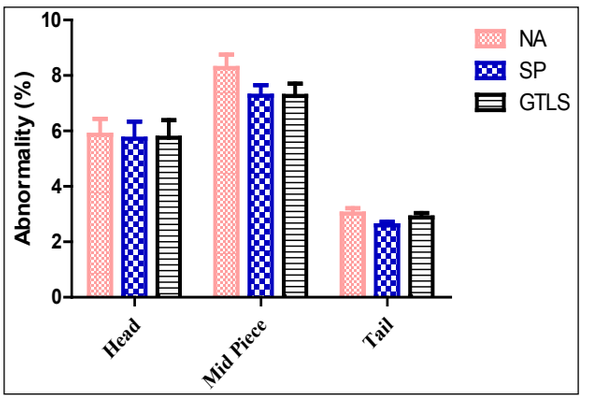

Fig. 4: Effect of antibiotics in semen extender on sperm abnormalities of breeding bulls. NA- No antibiotic; SP- Streptopenicillin; GTLS- Gentamicin, Tylosin, Lincomycin, Spectinomycin

The quality of both fresh ejaculate and frozen-thawed semen is crucial for effective artificial insemination. High-quality semen is characterized by minimal bacterial contamination and meets standard parameters for sperm motility and morphology. Bacterial contamination not only reduces sperm viability but also poses a risk of infections in the female reproductive tract, potentially affecting fertility. Recently, various alternatives to the traditional penicillin-streptomycin combination in egg yolk-based diluents have been explored to better control bacterial presence in frozen semen over long storage durations (Santos and Silva, 2020).

Yaniz *et al*. (2010) found that 13% of bacterial isolates from ram semen were resistant to strepto-penicillin, the most commonly used antibiotics in semen diluents. In this study, bacteria from bull semen samples showed higher sensitivity to gentamicin and spectinomycin as compared to other antibiotics. Notably, Staphylococcus species exhibited resistance to penicillin, emphasizing the greater effectiveness of the GTLS antibiotic combination over SP in controlling bacteriospermia. Semen treated with GTLS exhibited a significantly lower bacterial load than those treated with SP. Gentamicin and linco-spectinomycin, both broad-spectrum antibiotics, were particularly effective against both gram-positive and gram-negative bacteria, while tylosin showed effectiveness against mycoplasma (Santos and Silva, 2020). These findings are consistent with Andrabi *et al.* (2016), who reported a substantial reduction in bacterial load with GTLS over SP in frozen-thawed buffalo semen. The reduced efficacy of SP may be due to bacterial resistance, likely resulting from prolonged use of these antibiotics. The presence of microbes in frozen-thawed semen highlights the importance of implementing stricter control measures during collection, dilution, equilibration and cryopreservation, as bacteria can endure and remain dormant even in liquid nitrogen (Bielanski *et al.,* 2003) at temperatures as low as -196°C.

The better semen quality in GTLS treated group as compared to SP treated group as shown by improved frozen-thawed sperm motility, HOST, acrosome-intactness might be due to lowered microbial load as GTLS combination antibiotics were found sensitive against most of the isolated semen microbes and effectively controlled bacterial load in the semen. In the SP antibiotic combination, penicillin was found resistant to most of the isolated bacteria which may be responsible for poor bacterial load control in this group. Microorganisms present in semen can have both direct and indirect detrimental effects on sperm viability. Directly, they can cause sperm clumping, which disrupts normal sperm motility and reduces the release of acrosomal enzymes, leading to irreparable cellular damage. Indirectly, microorganisms can generate reactive oxygen species (ROS), triggering inflammation that further damages sperm cells. This combination of direct physical interference and oxidative stress exacerbates the decline in semen quality and fertility. These findings are consistent with the observations made by Moretti *et al.* (2009), highlighting the significant impact of microbial

contamination on semen integrity. The findings of the present study are consistent with those of Andrabi *et al.* (2016), who reported a significantly lower bacterial load in the GTLS group at both post-dilution and thawing stages when compared to the SP and control groups. This suggests that GTLS treatment effectively reduces bacterial contamination in semen, a result that aligns with previous studies. Similarly, Gerard *et al.* (1995) found that the addition of GTLS to bovine semen extenders led to a significant reduction in bacterial load and an increase in the number of spermatozoa with intact acrosomes, as compared to the control group. These studies collectively emphasize the potential of GTLS as an effective agent for improving semen quality by reducing bacterial contamination and preserving sperm function. Ngo *et al.* (2023) reported that gentamicin had positive effect on inhibiting bacterial growth during semen storage and preserved the quality of high-viability semen better than low-viability semen in boar.

The damage observed to the acrosome in the control group may be attributed to bacterial growth, which can adversely affect sperm function. The presence of microorganisms, particularly bacteria, in semen can directly interfere with the fertilization process by triggering an abnormal acrosome reaction. This reaction is typically induced through a calcium-dependent mechanism, as noted by Morell (2006) and Rennemeier *et al.* (2009). The acrosome, a vital structure on the spermatozoa, plays a crucial role in fertilization. Its integrity is vital for the acrosomal reaction, which must occur just before fertilization to allow the sperm to penetrate the ova. Damage to the acrosome can disrupt this process, impairing the sperm's ability to fertilize the ova and ultimately reducing fertility. Therefore, microbial contamination in semen can significantly hinder successful fertilization by compromising the acrosome's functionality.

In contrast to our findings, the semen quality of buffalo bulls was found to be superior in the group treated with SP. This improvement could be attributed to the dose related toxicity of gentamicin on sperm cells in the gentamicin-treated group, as noted by Akhter *et al.* (2008). Additionally, a recent study on Indian red jungle fowl by Rakha *et al.* (2024) demonstrated enhanced progressive motility, viability, and integrity of the acrosome and sperm membrane when a penicillin-containing extender was used. This was in comparison to control groups and other extenders containing antibiotics like gentamicin, streptomycin, kanamycin, and neomycin, which did not show the same benefits. Furthermore, Ali *et al.* (1994) observed that the viability of buffalo sperm cells was significantly improved when using extenders supplemented with SP or ampicillin, in contrast to those treated with gentamicin at refrigerated temperatures. These findings suggest that the choice of antibiotic extender plays a crucial role in maintaining sperm quality during storage. In the said study, no significant differences in sperm abnormalities, including head, mid-piece, and tail defects, across the various antibiotic-treated extender groups was reported. This finding aligns with previous research by Revell (2003), who indicated that the morphological integrity of sperm is not heavily influenced by semen preservation techniques or the type of extender used, as long as the processing methods are appropriate.

CONCLUSION

In conclusion, the results suggest that a combination of antibiotics, including Gentamicin, Tylosin, Lincomycin, and Spectinomycin, is more effective than the use of Streptomycin and Penicillin in controlling bacterial contamination and enhancing the post-thaw characteristics of bull semen. Specifically, these antibiotic combinations were shown to improve semen motility, sperm membrane integrity, and acrosomal intactness following cryopreservation. This reinforces the importance of selecting the right antibiotics in semen extenders to ensure optimal preservation and quality of sperm for artificial insemination.

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