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Contamination with *Proteus* and *Staphylococcus spp.* Effect on Physical Properties of Sheep Semen

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Abstract

THIS study was designed to determine the effect of bacterial contamination degree and type with *Proteus mirabilis* alone and *Staphylococcus aureus* alone and mixed of them species in ejaculation samples of fertile ram semen during storage quality. Semen was collected from six healthy mature rams using electro-ejaculator method, once a week, for the period 1/12/2022 to 1/2/2023. PH, motility (mass and individual), live sperm percentage and abnormal acrosomes of sperms were studied. Isolation and identification of *Proteus mirabilis* and *Staphylococcus aureus* species viable bacterial was recorded with routine bacterial detection work and molecular using PCR technique. Semen samples were divided into four groups: Group 1 (G1) was used as control without contamination. Group 2 (G2) was contaminated with *Proteus mirabilis*. The third group (G3) was contaminated with *Staphylococcus aureus*. The fourth group (G4) was contaminated with both *Proteus mirabilis* and *Staphylococcus aureus* together. Semen was examined after three days of incubation at 4C°. Statistically significant differences (P<0.05) group G1, G2 and G3 with G4 groups for all analysis parameters were recorded. Significant differences (P<0.05) between G1 with G2 in mass and individual motility, live percentage and abnormal acrosomes were recorded. Significant differences (P<0.05) between G1 with G3, G2 with G3 in live percentage and abnormal acrosomes were listed. Significant differences (P<0.05) between G2 with G4 in individual motility and abnormal acrosomes were noticed. We conclude that *Staphylococcus aureus* (alone) contamination will lower semen quality after cooling at 4C° and it decreases further more when the semen contaminated with *Proteus mirabilis*, the semen quality decreases was doubled with the mixture of both bacteria.

Keywords: *Proteus mirabilis*, *Staphylococcus aureus*, ram, semen quality, storage, semen contamination**.**

Introduction

Ovine species are the most important animals in Iraq. They have the highest economical degree in its production (skin, milk, wool and meat) [1]. Genetic improvement would increase when using a breeding technique such as artificial insemination [1]. Male reproductivity in sheep is affected by several factors [2]. Few researchers studied semen quality after bacterial contamination. Reproductive function has been affected by microbial contamination, motile sperm have been reduced, and reduction in acrosome reaction and sperm morphology would be affected due to inflammation response which is induced by bacterial infection as a result of the reactive oxygen species [3]. The presence of bacteria with semen does not indicate infection [4]. Bacterial contamination affects fertilization [5]. With bacterial contamination, morphology of sperms will be normal along with the presence of few inflammation cells [1]. The microbial infection will produce toxins as an indirect indicator for infection [4 and 6]. So the use of artificial insemination will reduce the microbial population associated with the ejaculate [1]. Some studies in Iraq investigated the infection of genital tract of females with semen contaminated were done [7 and 8]. The studies that had investigated *Staphylococcus aureus* did not investigate semen contamination were done before [9, 10 and 11]. The effect of bacteria on sperm quality has controversial

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theories, so the present study was designed to study the effect of *Proteus mirabilis*, *Staphylococcus aureus* bacteria individually and collectively on semen quality of the ram as ram semen usually is contaminated with bacteria that may affect the sperm vitality and fertilization efficiency. Semen quality is vital for successful artificial insemination in sheep. So, the effect of bacteriospermia on semen seems to play a role in male gametes damage.

Experimental

Period and location of the study: This study was conducted from 1/12/2022 to 1/2/2023. Rams were housed in Veterinary Medicine College, University of Kufa, Iraq.

Samples collection: Semen samples were collected from six healthy mature (2-3 years) males. Standardized healthy conditions (housing, feeding) were maintained during the study period. Total of 312 ejaculates were collected from all males, the semen collection made once a week using electro-ejaculator (Mini-Tube, Germany). For this study, the acceptable evaluation was 0.5-1.5 ml volume and 70% individual motility with less than 10% abnormal sperms. All samples that are contaminated with other types of bacteria than *Proteus mirabilis* and *Staphylococcus aureus* were excluded.

Extender preparation: Modified from Baiee *et al*. [15], the Tris-based extender was prepared of: 8% Low Density Lipoprotein (LDL), 2.4 gm tris, 1 gm fructose. 1.5 gm Citric Acid. The volume was adjusted to 100 ml with D.W. without antibiotics.

Bacteriological isolation and identification: The semen was tested for bacterial isolation and identification of all types of contamination. The primary step of isolating *Proteus mirabilis* and *Staphylococcus aureus* bacteria was done using blood agar. The Gram stain and biochemical tests were conducted to identify the bacteria. This was done following [12].

PCR Confirmation

Proteus mirabilis*:** PCR technique was used to indicate the presence of 16S rRNA gene of *Proteus mirabilis.** Pairs of primers were used for identification of gene (F 5`-GGAAACGGTGGCTAATACCGCATAAT-3` and R 5`-GGAAACGGTGGCTAATACCGCATAAT-3`). The reaction mixture consisted of: 1.5µl genomic DNA, 10µl PCR master mix, 0.75µl of each primer and the final volume was adjusted to 20µl).

Denaturation steps were at 94 Cº for 4 minutes and 40 seconds respectively followed by the annealing step at 58 Cº for 60 seconds. The extension step was at 72 Cº for 20 seconds. Final extension was done at 72 Cº for 10 minutes. The total number of reaction cycles was 30. Then electrophoresis processed in 1.5% gel agarose at 100v for 30 minutes and ethidium bromide under UV for visualization. This was done according to Zhang *et al*. [13].

Staphylococcus aureus detection was performed by DNA isolates for the 16S RNA gene using the primers (F 5'-GGTCTTGCTGTCACTTATAGATGG-3' and R 5'-CGGAAGATTCCCTACTGCTG-3'). The 50µl reaction mixture contained (1µl of DNA, 20µl of each primer, 1.5µl master mixture, and 7.5 µl nuclease free water). Denaturation started at 94 Cº for 5 min., then 94ºC for 30 second, Annealing step 56 Cº for 30second, extension step 72 Cº for 90 second and the final extension with 72 Cº for 10 min with 30 cycle in total. The gel electrophoresis confirmation conducted with 2% agarose solution stained with ethidium bromide and visualized under UV trans-illuminator. This was done according to Al-Musawi *et al*. [14].

Experimental design: Semen were divided into four groups (G1) (Control): Contamination free, G2: *Proteus mirabilis* contaminated, G3: *Staphylococcus aureus* contaminated and G4: A mixture of *Proteus mirabilis* and *Staphylococcus aureus* contamination.

Semen analysis: In line with Yaniz *et al*. [16], semen analysis was conducted after 3 days of incubation at 4 Cº then pure bacterial colonies were added to the experimental groups. Prior to induced contamination, semen was evaluated twice for pH, sperm mass motility and sperm individual motility. pH was measured using litmus paper; Under 100X magnification, Sperm mass motility was scaled from 0-100%. Under 400X magnification, semen placed on 37 Cº slides was examined for individual motility which was calculated based on progressive motility of sperms. Eosin-nigrosin stain methods were used to determine the sperms livelihood status and the fast green stain method was used to estimate the percentage of abnormal acrosome.

Statistical analysis: means ± SE, of samples was examined by Chi square using SAS [17].

Results and Discussion

The number of negative bacterial contamination in collected semen reach 19 (6%) from all collected semen. The number of contaminated semen with *Proteus mirabilis* only reach 18 (5.5%), and the number of contaminations with *Staphylococcus aureus* only reach 35 (11%), while the number of mixed contamination with both types of microbes reach 9 (3%).

At $P<0.05$ significant differences, a comparison for the parameters pH, mass and individual motilities %, live sperms %, and abnormal acrosomes % between (G1), (G2), (G3) and (G4) , table 1 shows significant differences in pH between G1 and G3 with G4, but no significant differences within G1, G2

and G3. Also no significant differences had been recorded between G2 and G4 (Table 1).

Mass motility tests showed that there were significant differences (P<0.05) between non-contaminated semen (G1), semen contaminated with *Proteus mirabilis* (G2) and semen contaminated with a mixture of *Proteus mirabilis* and *Staphylococcus aureus* (G4) (Table 1). Semen contaminated with *Staphylococcus aureus* (G3) showed significant difference (P<0.05) compared to the semen contaminated with the mixture of *Proteus mirabilis* and *Staphylococcus aureus* (G4) (Table 1). Individual motility tests indicated that there were significant differences (P<0.05) between non-contaminated semen (G1) and the semen contaminated with *Proteus mirabilis* (G2) and with the mixture of *Proteus mirabilis* and *Staphylococcus aureus* (G4) (Table 1). Also, semen contaminated with contaminated with *Proteus mirabilis* (G2) and *Staphylococcus aureus* (G3) showed significant differences (P<0.05) compared to semen contaminated with the mixture of *Proteus mirabilis* and *Staphylococcus aureus* (G4) (Table 1). Live sperms test indicated that there were significant differences (P<0.05) between non-contaminated semen (G1) and all other groups (Table 1). Semen contaminated with *Staphylococcus aureus* (G3) showed significant differences (P<0.05) with semen contaminated with *Proteus mirabilis* (G2) and semen contaminated with mixed *Proteus mirabilis* and *Staphylococcus aureus* (G4) (Table 1). Abnormal acrosomes tests showed that semen contaminated with the mixture of *Proteus mirabilis* and *Staphylococcus aureus* (G4) has a significant increase compared to all groups (Table 1). Also, semen contaminated with *Proteus mirabilis* (G2) has a significant increase (P<0.05) than semen contaminated with *Staphylococcus aureus* (G3) and non-contaminated semen (G1) (Table 1). There was a significant increase (P<0.05) in abnormal acrosomes defect in semen contaminated with *Staphylococcus aureus* (G3) and non-contaminated semen (G1) (Table 1).

This study is the first trait to investigate bacterial contamination with *Proteus mirabilis*, *Staphylococcus aureus* and their mixture in rams' semen in Iraq. Previous records of isolating microbes from sheep semen were *E coli, Proteus mirabilis, Enterobacter cloacae, Staph epidermis and Staph aureus* [16 and 18].

Fresh semen may contain some of non-pathogenic bacteria [19. The heavy presence of microbial agents results in infertile mating [19]. Microbial contamination may occur during semen collecting or storage processing [20]. Such microorganisms may cause the risk of disease infection via semen leading to bacteraemia, viraemia and genital tracts infections [21]. Semen contamination with microbial agents will reduce sperm count, morphology and motility [22] which agree with this study results. Presence of microorganisms with semen leads to decreasing the fertility rate [19]. This appears to be fit with herein study. Microorganism (bacteria, fungus and virus) presence with semen will lower its quality and may transmit the pathogens to next generation [23]. There is a positive correlation between bacterial count and the decrease in sperm motility and its live percentage [20, 24 and 25]. Negative effect can be direct through the presence of the bacteria or it can be indirect due to the toxins production [4, 6, 26 and 27]. This was demonstrated in this study where the direct negative effect on semen was recorded. Presence of bacteria will produce a byproduct that kills sperms [26 and 28]. In agreement with this study, Tvrda *et al*., [29] stated that bacterial contamination of semen is important factor for the decreased sperm vitality. Some bacteria contain lipopolysaccharides (LPS) in their walls which will be released after the death of the bacteria damaging the sperms. Furthermore, these bacteria cause infection and diseases in served female [30]. Contamination with mixed bacteria would have more adversely affected than single contamination. This was also demonstrated in this study where the mixed infection decrease fertility more than infection with single flora or pathogenic bacteria. The resistant *S. aureus* strains at rate reach 94.3% [31]. So, the infection with such bacteria may lead to decrease fertility.

Conclusions

Researchers concluded that rams' semen contamination with *Staphylococcus aureus*h as a negative effect on its quality and it is more adverse if the contaminant was *Proteus mirabilis.* However, the effect was doubled when the contamination was with the mixture of the two bacteria together which was represented in the poor semen characters (Decrease in pH, lower mass and individual motility and reduced live percentage with high acrosomal abnormalities). All these would certainly lead to low semen quality.

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Conflicts of interest

There are no conflicts to declare.

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TABLE 1. Mean ± SE values of rams' semen pH, mass motility (%), individual motility (%), live sperm (%), abnormal acrosomes between non-contaminated (G1), semen contaminated with *Proteus mirabilis* (G2), semen contaminated with *Staphylococcus aureus* (G3) and semen contaminated with a mixture of *Proteus mirabilis* and *Staphylococcus aureus* (G4) bacterial species after 3 days of incubation at 4C°.

Parameters Tested	(G1)	(G2)	(G3)	(G4)
pH	6.60 ± 0.04^{A}	6.49 ± 0.04^{AB}	6.56 ± 0.03^{A}	6.41 ± 0.03^{B}
Mass Motility (%)	75.2 ± 1.65^{A}	60.6 ± 2.67^{BC}	69.4 ± 1.89^{AB}	57.5 ± 2.93^{C}
Individual Motility (%)	76.85 ± 0.48^{A}	62.33 ± 2.87^{B}	71.62 ± 2.62^{AB}	50.63 ± 1.84^{C}
Live Sperm (%)	76.5 ± 0.31^{A}	61.59 ± 2.75^{C}	71.72 ± 1.99^{B}	55.83 ± 1.65^{C}
Abnormal acrosome (%)	8.51 ± 0.25^{D}	12.72 ± 0.33^{B}	10.56 ± 0.41^{C}	14.52 ± 0.32^{A}

- Means ± SE of each parameter in the same row.
- Different as a and b tested using Chi-square, the significant at $P<0.05$.

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التلوث بالمتقلبة والمكورات العنقودية وتأثيرها على الصفات الفيزيائية للسائل المنوي للكباش

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هذه الدراسة أنجزت لتحديد درجة تأثير التلوث بالأنواع المتقلبة الشائعة والمكورات العنقودية الذهبية لوحدهما أو بشكل مختلط في عينات القذفات للكباش خلال مراحل الخزن. السائل المنوي تم جمعه من ستة كباش بالغة بأستعمال القاذف الكهربائي مرة أسبوعياً لفترة التجربة الممتدة من 2022/12/1 إلى 2023/2/1. درجة الحموضة، الحركة (الجماعية والفردية)، نسبة الحيامن الحية وتشوه الأكروسوم للحيامن تم دراسته. عزل وتشخيص المتقلبة الشائعة والمكورة العنقودية الذهبية تم بواسطة الطرق الروتينية والجينية بأستعمال طريقة تفاعل سلسلة البلمرة. عينات السائل المنوي تم تقسيمها إلى أربعة مجاميع: المجموعة 1 أستعملت كسيطرة خالية من التلوث. المجموعة 2 كانت ملوثة بالمتقلبة الشائعة. المجموعة الثالثة كانت ملوثة بالمكورة العنقودية الذهبية. المجموعة الرابعة كانت ملوثة مع كلا الجرثومتين معاً (المتقلبة الشائعة والمكورة العنقودية الذهبية). تم فحص السائل المنوي بعد ثلاثة أيام من الخزن في درجة حرارة 4 مئوية. الفروق الأحصائية المعنوية على مستوى أقل من 5% للمجموعة 1، 2 و 3 مع المجموعة 4 في كل المعايير المدروسة. الفروق المعنوية على مستوى أقل من 5% بين المجموعة 1 و 2 في الحركة الجماعية والفردية، نسبة الحيامن الحية والتشوه في الأكروسوم تم تسجيلها. الفروق المعنوية على مستوى أقل من 5% ما بين المجموعة 1 و 3 من جهة وما بين المجموعة 2 و 3 في نسبة الحيامن الحية وتشوه الأكروسوم تم تسجيلها أيضاً. الفروق المعنوية على مستوى أقل من 5% بين المجموعة 2 و 4 في الحركة الجامعية وتشوه الأكروسوم تم ملاحظتها. نستنتج من ذلك أن المكورة العنقودية الذهبية لوحدها سوف تقلل من كفائة السائل المنوي للكباش بعد تبريده في درجة حرارة 4 مئوي وسوف يقل أكثر عندما يتلوث بالمتقلبة الشائعة، وأن كفاءة السائل المنوي سوف تقل بنسبة الضعف مع التلوث المختلط بكلتا الجرثومتين.

الكلمات الدالة: المتقلبة الشائعة، المكورة العنقودية الذهبية، الكباش، كفاءة السائل المنوي، الخزن، تلوث السائل المنوي.