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Bacterial Contamination of Imported Bulls Frozen Semen

H. B. Najee, A. M. Al-Shawii and L. Y. Abd- Al Rahman
College of Veterinary Medicine/ University of Baghdad

Abstract

One hundred frozen semen straws (0.5 ml), have been taken from imported bulls, the frozen semen examination as well as evaluation in addition to bacteriological evaluation whether counting, isolation and antibiotic sensitivity, the results in current study showed all bacterial isolates were identified by using different biochemical tests and API–20 E system that used later to confirm identification, all isolated bacteria found in imported frozen semen were gram negative.45 bacterial isolates in imported frozen semen distributed in 42 isolates *Stenotrophomonas maltophilia* and 3 isolates *Pseudomonas aeroginosa*, antibiotic susceptibility test for isolated bacteria by using 14 single antibiotic discks of commonly used drugs, most isolated *Steno. maltophilia* was susceptible to Ceftazidime, Ciprofloxacin, Cefotaxime, Gentamicin, Amikacin, Vancomycin, Erythromycin, Chloramphenicol and Tetracyclin. The results of current study showed individual movement 61.3±2.00, viability of sperm 76.2±2.06, percentage of dead sperms 23.8±2.06, and abnormal sperms 13.54±0.67.

التلوث البكتيري في قصبات السائل المنوي المستورد للثيران

هيفاء أبعاوي ناجي، أمل ماجد الشاوي ولقاء يونس عبد الرحمن
كلية الطب البيطري/ جامعة بغداد

الخلاصة

أخذت 100 قصبه من السائل المنوي المجمد المستورد 0.5 مل, أجريت فحوصات تقييم السائل المنوي واختبارات العد والعزل الجرثومي على العينات واختبار فحص الحساسية للمضادات الحيوية المختلفة. كل الجراثيم التي عزلت شخصت, وأجريت الفحوصات الكيموحيوية التقليدية والتشخيص بنظام API–20E لتشخيص الأنواع الجرثومية المعزولة. كانت جميع الجراثيم المعزولة من عينات السائل المنوي المجمد المستورد سالبه لصبغة كرام, خمسة وأربعون عزلة جرثومية عزلت من السائل المنوي المستورد موزعه في 42 عزله من*Stenotrophomonas maltophilia* و3 عزلات من *Pseudomonas aeruginosa*. اختبرت الحساسية للجراثيم المعزولة وتم استخدام 14 نوع من الأقراص المفردة للمضادات الحيوية الأكثر استخداما, أغلب عزلات *Steno. maltophilia* كانت حساسة للـ Ceftazidime, Ciprofloxacin, Cefotaxime, Gentamicin, Amikacin, Vancomycin, Erythromycin, Chloramphenicol وTetracyclin, من ناحية أخرى كانت جميع الجراثيم المعزولة مقاومه بنسبة 100% لكل من الـ penicillin وStreptomycin وAmoxicillin. بينت نتائج الدراسة الحالية للعينات المستوردة ان الحركة الفردية 61.3±2.00 وحيوية الحيامن 76.2±2.06 والنسبة المئوية للحيامن الميتة 23.8±2.06 أما النسبة المئوية للحيامن المشوهة فكانت13.54±0.67.

Introduction

Artificial insemination has facilitated the choice of using the best possible bulls of proven fertility in improving the genetic makeup of the cattle population thus conveying

to the primary goal for breeding; to increase the productivity and the profitability of a particular commercial herd by increasing the number of offspring produced by selected genetically superior bulls. The use of AI made also possible to measure the performance of large numbers of progenies born after AI of many females using sperm doses from a single bull, thus allowing the accurate selection of bulls with desirable characters (1). Sperm cryopreservation contributes to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro fertilization, AI with frozen semen is essential in breeding and selection schedules contributing to increase production of domestic species. These schemes were well developed in dairy cattle (2). Presumed mechanisms of infection, causing infertility are the following: (a) Bacterial attachment to sperm; (b) an immobolizing factor produced by some bacteria; (c) immune system recruitment, and (d) alteration of glandular function (3). The pathogenic bacteria in the ejaculates can induce a defect in semen parameters, such as reduce sperm count, poor morphology and motility (4). It is already known that these parameters play a vital role in the fertility (5). The aim of study to know the types of bacteria present in frozen semen used for A.I.

Materials and Methods

One hundred imported frozen semen samples was taken and make on these samples many examinations such as:

- **Frozen semen evaluation:** Taken out of frozen semen straws after storage and has been found important to thaw it by placing it (with effective separation from the water) in water bath at 37c^{o} for 30 sec. After drying, both sides of frozen semen straw has been broken, in order to take a drop of semen after neglecting the first drop, and then make the following tests: Bacterial isolation, pH, Individual movement and The percentage of viability and morphologically abnormal sperm.

1. Bacterial isolation: Samples were taken from frozen semen and cultured on the nutrient broth, and another sample was cultured in tryptic soya broth, samples then were cultured in two ways:

A. Aerobically: Placing the samples were cultured in nutrient broth in incubator at temperature of 37c degree and for a period of 24-48 hours, then were transported the growth from nutrient broth on three kinds of culture media (Blood agar, MacConky agar, nutrient agar) and cultured by streaking method, then placed in incubator at 37ºc for a period of 24-48 hours, then was diagnosed types of bacteria that appeared on the culture media by using Gram stain and biochemical tests and by using Api-20E system.

B. Facultative anaerobic: Placing the samples were cultured in tryptic soya broth in candle jar for 7-14 days in order to note the presence of bacterial species that need for their live to 10% of carbon dioxide (CO_2), such as *Brucella* and *Vibrio*, after the incubation were transported growth in the broth among Special media such tryptic soya agar and Brucella agar and placed in candle jar and put it in incubator under 37°c for 7-14 days until the colonies is a appear, diagnosis of the colonies by the way (6).

- **Bacterial Count:** Bacteria were counted by the way plate count method known as Miles and Misra (7). Make a series of tenfold dilution to semen sample mixing 0.9 ml of phosphate saline solution with 0.1 ml of semen sample to get dilution 1:10, and continuous to make series of dilution, then cultured the final three dilution on MacConky agar and then using a Pasteur pipette and incubation the petri dishes in incubator at 37°c for 24 hours and then calculate the number of colonies of bacteria.
- **Antibiotics sensitivity test**: According to 8 plates were prepared with Muller Hinton Agar, 3-4 similar colonies were selected to prepare pure culture, these colonies were transferred into about 5ml of trypticose soya broth incubated at 37 ^{O}c

for 2-8 hrs until light to moderate turbidity develops, turbidity adjusted by using MacFarland tube to 1.5 $\times 10^8$ cfu/ ml Dipasterile non toxic cotton swab on a wooden applicator into the standardized inoculums and rotate the soaked swab firmly against the upper inside wall of the tube to express excess fluid ,streak the surface of the plate with the swab three times, allow the inoculums to dry for 5-15 minutes with lid in place, antibiotics discs which include (Penicillin, Gentamycin, Streptomycin, Amoxicillin, Chloramphenicol, tetracyclin, Erythromycin, Ceftriaxone, Tobramycin, Vancomycin, Amikacin, Ceftazidime, Cefotaxime, and Ciprofloxacin) these disc were fixed by using aseptic technique and then incubated immediately at 37°c and examine after 24 ^{o}c, the complete inhibition zone was recorded.

2. pH**:** pH was evaluated for frozen semen by mean of special tape runway from 5.5 to 9.5 is intended for this purpose. Evaluated the pH of frozen semen revenue to (AI) center, local Vet. Clinics and imported.
3. **Individual motility (%):** Estimated percentage of the movement of individual sperm frozen semen taken from the (AI) center and local Vet. Clinics and imported in the following manner: A drop of thawed semen was placed over a warm slide and covered by cover slide. using light microscope 10 x 40 (400), several fields were examined to estimate the percentage of individual motility of sperm, which was assessed according to the method reported by (9). Considered the individual active progressive movement forwards the guide of the quality of screened semen.
4. **The percentage of viability and morphologically abnormal sperm:** Were measured as follows, a drop of thawed semen was mixed with drop of eosin stain and two drops of nigrosin stain, A thin smear of semen eosin- nigrosin mixture was done by using other slide and left to dry. Two hundred sperm were counted to calculate the percentage of viability sperm and morphologically abnormal sperm as in the following equation: The most important characteristic of the semen of bulls with high fertility is the low percentage of abnormal sperms, the percentage of abnormalities more than 30% indicate poor quality of semen and low fertility**.**

$$\text{Sperm Viability} = \frac{\text{No. of viable sperms}}{\text{Total No. of sperms}} \times 100$$

$$\text{Sperm Abnormality} = \frac{\text{No. of abnormal sperms}}{\text{Total No. of sperms}} \times 100$$

Results and Discussion

- **Cultural results:** Colonies were obtained pure and separate through the culture, and growing on nutrient agar medium, blood agar medium, tryptic soya broth medium and then transferred on differential media such as MacConky agar, manitol salt agar characterization of colonies depends on the shape of colonies: The growing colonies on the MacConky agar lactose-non fermented were two types of bacteria, first Colonies was yellow or greenish yellow with Ammonia odor, on blood agar some strains may produce slight beta-haemolysis. The second one, colony was large, low, round, convex and smooth, sometimes surrounded by serrated growth, pale bluish green in color with a grape-like odor, some strains hemolyze blood.
- **Biochemical tests results:** Table (1) showed the results of some biochemical tests diagnostic and differential to isolated bacteria: The isolates can be biochemically distinguished from the known species and confirm the specific characteristics of each bacterial isolate. Furthermore, it has been possible to develop a practical laboratory test based on the characterized biochemical activities and can be devised a diagnostic strategy for the specified species of a bacteria (10). The biochemical

properties of the organism recorded in this study are the same as obtained by other workers (11,12,13). Our findings are partially agreed with the observations made by Collins and Patricia (14). The different pigments, which were produced on various media exhibited by *Pseudomonas aeruginosa* was considered due to high oxidative activity. This oxidative activity is the sole property, which is responsible to differentiate various species of the same genus (10).

Table (1) Results of biochemical tests for isolated bacteria

Type of bacteria		*Stenotrophomonas maltophilia*	*Pseudomonas aeruginosa*
Lactose fermentation MacConkey		NLF	NLF
Indol		-	-
Citrate		+	+
Urease		-	v
Phenylalanine		-	-
Gelatin liquefaction		+	+
Swarming		+	-
Motility on SIM		+	+
TSI	Reaction	ALK/ALK	ALK/ALK
	H_2S	-	-
	Gas	-	-
Oxidase		-	+

NLF=no lactose fermentation **ALK=alkaline reaction**
LF=lactose fermentation **+=positive result**
V= variable reaction between species **- = negative result**

- **The results of biochemical tests by using API-20E system:** The API-20E system, is one of the most important diagnostic tests and more accurate diagnosis of most of the gram negative bacilli bacteria conformity with the results of conventional biochemical tests as shown in table (2).

Table (2) Biochemical tests results for Api-20E system to diagnosis isolated bacteria

APA-20E	*Stenotrophomonas maltophilia*	*Pseudomonas aeruginosa*
ONPC	-	-
ADH	-	+
LDC	+	-
ODC	-	-
CIT	+	+
H_2S	-	-
URE	-	-
TDA	-	-
IND	-	-
VP	-	-
GEL	-	+
GLU	+	-
MAN	-	-
NO	-	-
SOR	-	-
RHA	-	-
SAC	-	-
MEL	-	-
AMY	-	-
ARA	-	-
OX	-	+

- **The results of bacterial isolation:** One hundred frozen semen samples have been tested, the bacteria isolated from imported frozen semen samples *Stenotrophomonas maltophilia* 42% and *Pseudomonas aeruginosa* 3%, while the samples have no

growth were 55%. Piasecka-Serafin (15) was first to demonstrate the possibility of translocation of bacteria from infected semen pellets to sterile ones in LN. From the results of bacterial isolation in different studies showed that presence of many types of bacteria in frozen semen according to many researchers(16,17,18,19 and 20). Different studies demonstrate that presence of bacteria in seminal fluid has been associated with infertility (21). Changes in sperm parameters that could account for this effect include reduced cell counts reduced motility or morphology alteration (22). *Stenotrophomonas maltophilia* was the most prevalent bacterial strain detected in cryopreserved samples (19). In current study, the percentage of *Stenotrophomonas maltophilia* more than other types of bacteria that may be return to it widely distributed including moist hospital environments as a contaminated bacteria (23) and also it found in liquid nitrogen (24). *Pseudomonas aeruginosa* is a frequently isolated contaminant of bull semen, (19) *Pseudomonas aeruginosa* can cause serious genital diseases in breeding bulls (25) and interfere with the sperm cell viability and probably lower the conception rate too (26, 27), Also if *Pseudomonas aeruginosa* is transmitted to the females genital tract at the time of breeding, it can result in infectious endometritis and associated subfertility (28).

- **Bacterial count:** The results of bacterial count in samples of imported frozen semen 10.43±1.05, 12.49±0.03 for bacteria *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* respectively.
- **The results of sensitivity test to antibiotics:** Susceptibility test Showed that most isolates of bacteria which was isolated from frozen semen resistance to most antibiotics used, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* isolated from samples of imported frozen semen resistant to Penicillin, Streptomycin and Amoxicillin in percentage 100%. Bacterial resistance to antibiotics as follows: *Stenotrophomonas maltophilia* resistant to Cefotaxime 33.3%, Gentamycin 40.5%, Tobramycin 95.2%, Amikacin 66.7%, Vancomycin 50%, Erythromycin 16.7%, Tetracycline 4.8%and Ceftriaxone 90.5%, while *Stenotrophomonas maltophilia* was sensitive 100% for Ceftazidime, Ciprofloxacin and Chloramphenicol. *Pseudomonas aeruginosa* resist 100% for each of Gentamycin, Tobramycin, Amikacin, Erythromycin, and Ceftriaxone. The resistance percentage for each of the Ceprofloxacin, Cefotaxime 33.3%, and 66.7% for Tetracyclin,Vancomycin and Chloramphenicol as in table (3). Gentamycin, being broad spectrum, are more effective against gram-positive and negative bacteria (29, 30). Another explanation could be that some of the organisms, due to excessive use of the drug, had mutated and became resistant (30, 31). Alternatively, the sensitivity of SP (Streptomycin and Penicillin) against the microorganisms was reduced. Most bacterial isolates in current study were susceptible to Gentamycin, this results agreed with the results obtained by others (32, 33), this might be attributed to the action of Gentamycin that return to aminoglycosides group. The resistance *Pseudomonas* to Streptomycin and Penicillin were also reported by Palli *et al*. (34) and Aleem *et al*. (35). Lazarevic *et al*. (36) reported the wide uses to Penicillin and Amoxicillin in treatment may leads to high resistance to these antibiotics, also the randome of antibiotics causes a high resistance percentage (37).

Table (3) The results of susceptibility test to antibiotics for isolated bacteria from imported frozen semen

Type of bacteria		*Stenotrophomonas maltophilia*	*Pseudomonas aeruginosa*
Penicillin	No.	42	3
	%	100%	100%
Streptomycin	No.	42	3
	%	100%	100%
Ceftazidime	No.	0	0
	%	0%	0%
Ciprofloxacin	No.	0	1
	%	0%	33.3%
Cefotaxime	No.	14	1
	%	33.3%	33.3%
Gentamicin	No.	17	3
	%	40.5%	100%
Tobramycin	No.	40	3
	%	95.2%	100%
Amikacin	No.	28	3
	%	66.7%	100%
Amoxicillin	No.	42	3
	%	100%	100%
Vancomycin	No.	21	2
	%	50%	66.7%
Erythromycin	No.	7	3
	%	16.7	100%
Chloramphenicol	No.	0	2
	%	0%	66.7%
Tetracyclin	No.	2	2
	%	4.8%	66.7%
Ceftriaxone	No.	38	3
	%	90.5%	100%
Total	No.	42	3

- **Some characteristics of frozen semen.**

1. **pH:** The results of current study showed that the pH of the frozen semen tended to the neutral 7.01± 0.03 table (4). Similar observations have been reported (38) as a results of current study, the pH ranged between 6.5-7.0, where researchers noted that a higher metabolic rate is expected when the pH of semen is maintained near neutrality 7.0. They suggested the importance of diluting semen in a buffered medium that resist changes in pH, so that maximum fertile life of the semen can be maintained (38).
2. **Percentage of sperm motility (%):** Sperm motility is the most parameter frequently used to measure sperm viability of the ejaculate and during the process of cryopreservation, and it has been related to fertility post artificial insemination (1). Sperm motility percentage of imported frozen semen 61.3±2.00 table (4). Decrease the percentage of sperm motility in the study of (1, 39) 52.6±2.4%, 50%, respectively compared with current study, that decrease might be due to the difference in breed, age, management-method of bulls. As well as, the percentage of sperm motility (forward movement) affected by freezing process because the spermatozoa exposures to different temperatures and PH with a long of neutral and freezing processes (40). Also the packaging, handling and freezing the semen in straws considered as an important causes of low quality semen (41).
3. **Percentage of sperm viability (Live and dead sperms %):** Semen with more than 30% initial dead spermatozoa may not be suitable for storage and freezing (42). Differential staining techniques have been used for determination of live and dead

spermatozoa (43). The results showed that the viability of imported frozen semen was 76.2±2.06, (Table 4). The values of live spermatozoa in the current study are higher than the average values recorded by other worker (44, 42) 61.6±2.59 and 43.5±1.46, respectively. This difference might be due to semen which has been frozen is difficult to assess with eosin as cryoprotectants, such as glycerol enhance penetration of the vital stain into the cell, thereby giving artificially high percentage of dead cells. Bearden and Fuquay (38) demonstrated the sperm viability which have a high correlations with fertility.

4. **Percentage of morphologically abnormal sperms:** Sperm morphology indicates normality or eventual deviations of the spermatogenesis and sperm maturation in the epididymis (1). The proportion of Morphologically abnormal spermatozoa in semen correlated negatively with fertility (45, 46). The result of abnormal sperms in present study was 13.54±0.67, Table (4). The finding of present study was agreed with many investigators observed the incidence of spermatozoa with abnormal morphology in fertile bull to be 0-18% (47). The values of abnormalities recorded in the present study are lower than the average reported by Zalzala (44) 34.1±2.24, the differences among the bulls of the studies which may be due to variation in general healthy body condition, their scrotal circumference, breed, age, genetic factors and the functions of accessory sex glands (48, 49). Moreover, collection frequency, precollection sexual stimulation, feeding regimen and climatic conditions can also influence the sperm abnormalities (50).

It was concluded from this study that the imported bull frozen semen might be contaminated by several types of bacteria and influence the semen pictures.

Table (4) Some characteristics of frozen semen

Characteristic	Imported straws (100)
pH	7.01±0.03
Individual motility (Motile sperm %)	61.3±2.00[ab]
Live sperms %	76.2±2.06[b]
Dead sperms %	23.8±2.06[a]
Abnormal spermatozoa %	13.54±0.67[a]

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