Isolation of *Moraxella bovis* from frozen bovine semen and determination of microbial load

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Abstract In the present study, isolation of *Moraxella bovis* was done from the microbiological examination of frozen semen straws from clinically healthy twelve Jersey and one Jersey-cross bull supplied by a semen laboratory. The organism was identified on the basis of colonial morphology and biochemical characteristics. Further, viable cell count of the bacterium was studied in detail in the semen sample.

Keywords Semen · *Moraxella bovis*.

Introduction

The bacterial contaminants of semen have been a major concern for the most semen production laboratories as contamination adversely affects the semen quality [1] and subsequently the fertility [2, 3]. Artificial insemination is an important procedure for increasing animal production and frozen bovine semen is most commonly used for the same. Semen (with antibiotics incorporated in it during the processing stage) is frequently contaminated by bacteria that survive the storage temperature of -196°C in liquid nitrogen and therefore acquire a certain level of resistance to the antibiotics [4]. This further account for the contamination of approximately 50 per cent of frozen semen samples [5]. The bacterial contamination of semen is important determinant of semen quality as it incites the production of macrophages and polymorphonuclear granulocytes as the first line of defense against these organisms, and this in turn leads to the production of reactive oxygen species (ROS), which are detrimental for sperm motility and viability. A variety of bacterial agents like *E. coli*, *Pseudomonas* spp., *Staphylococcus* spp. etc. may be isolated from frozen semen samples which may contaminate the semen during collection, processing and at the time of storage of the semen straws. The present work describes the isolation of an unusual isolate of *Moraxella bovis* from frozen bovine semen samples.

Moraxella bovis is a Gram-negative bacterium implicated in the pathogenesis of infectious bovine keratoconjunctivitis (IBKC), a disease causing significant economic losses in cattle industries worldwide [6, 7, 8]. *Moraxella bovis* also occurs in the eyes and nasal cavity of cattle asymptomatic with bovine keratoconjunctivitis (IBKC), whereas, *Moraxella phenylpyruvica* is associated with female genital tract infections [9]. *M. bovis* is an opportunistic pathogen

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whose virulence is influenced by both host and environmental factors. It is noteworthy that cattle are the primary natural reservoir for *M. bovis*.

In this study, 13 semen straws received from Sperm Station, Palampur (ISO-9001), Himachal Pradesh were examined for microbiological isolation and concurrent determination of bacterial load. Twelve Jersey bulls and one Jersey-cross bull, ranging between 3–5 years of age, clinically normal, were used as semen donors. All the standard procedures from semen collection to its storage in liquid nitrogen are followed in strict aseptic conditions and Streptomycin sulphate (100 µg/mL) and Penicillin (100 IU/ml) are added in the semen extender during the processing stage. Frozen semen straws from these animals were inoculated onto blood agar (BA) with 5% defibrinated sheep blood, MacConkey Lactose agar (MLA) and Sabouraud-dextrose agar (SDA) plates after thawing. After inoculation, BA and MLA plates were incubated aerobically at 37°C for 24–48 h and examined. The SDA plates were kept at 25°C for at least seven days. The microbes were identified by macro-microscopical morphology and biochemical characteristics. Catalase, oxidase, gelatinase, glucose, nitrate reduction and motility tests were used for identification of isolated strains.

Out of thirteen semen samples that were processed only one bacterial isolate could be recovered from bull no., JY- 3334 in pure culture. No fungal growth was however, observed. After 48 h of incubation, small, grayish-white, round colonies were observed along with narrow zone of complete haemolysis. No growth on MLA was seen. Gram stain smears from colonies on blood agar showed the presence of Gram-negative rods, characteristically in pairs. The catalase, oxidase and gelatinase tests were positive for the culture. The isolate was biochemically negative

for nitrate reduction and glucose utilization. The organism was non-motile and colonial growth autoagglutinated when suspended in saline. On the basis of the colonial morphology and biochemical reactions the culture was identified as *Moraxella bovis* [10].

Viable counting technique, was also applied to detect the number of viable bacteria of *Moraxella bovis* that were capable of multiplication with the production of visible colonies on or in the agar media. For this purpose pour plate method [11] was used. A ten fold serial dilution of bacterial suspension was made to find the number of bacteria, per mL of original sample. 0.1 mL of inoculum was mixed thoroughly with molten nutrient agar, previously held in water bath at 50°C. Separate plates were used with each dilution and agar was allowed to set and then incubated at 37°C for 24–48 h. Plates inoculated with sample dilutions yielding between 30–300 colonies per plate were read and the number of bacteria per mL of sample were calculated using the formula:

$$\text{Number of bacteria /mL of sample} = \frac{\text{No. of colony forming units (cfu's)} \times \text{dilution} \times 10}{1}$$

Pour plate method revealed 130 cfu's of *Moraxella bovis* in 10^{-2} dilution thus giving a microbial load of 130,000 bacteria / mL (Table 1). The findings of the present study may be compared with the previous reports [12] ($1.1 \times 10^2 \text{ mL}^{-1}$ in crossbred bull) whereas, some other workers have reported significantly higher bacterial load in semen samples ($8.1\text{--}390.4 \times 10^2 \text{ mL}^{-1}$ in frozen semen of Holstein stored for 15 days, $1.0\text{--}50.0 \times 10^2 \text{ mL}^{-1}$ in buffalo semen) [13, 14]. The significantly lower bacterial load from the frozen semen samples in the present study may be due to maintenance of strict aseptic conditions at the time of semen collection and processing during the entire steps from collection of semen to making of straws.

Table1 Details of number of CFU's obtained in different dilutions by pour plate method

S. No.	Semen straw no.	Bacterial isolates	CFU's			
			10^{-1}	10^{-2}	10^{-3}	10^{-4}
1.	JY-12	-	-	-	-	-
2.	JY-43	-	-	-	-	-
3.	JY-50	-	-	-	-	-
4.	JY-52	-	-	-	-	-
5.	JY-80	-	-	-	-	-
6.	JY-378	-	-	-	-	-
7.	JY-1544	-	-	-	-	-
8.	JY-1545	-	-	-	-	-
9.	JY-1567	-	-	-	-	-
10.	JY-1586	-	-	-	-	-
11.	JY-3295	-	-	-	-	-
12.	JY-3334	<i>Moraxella bovis</i>	>300	130	23	5
13.	CBJY-221	-	-	-	-	-

Bacterial contamination of semen may occur at any time right from collection through the various steps involved in preparing frozen straws. Additionally, the procedure involving part human and part mechanized operations can lead to contamination at various levels. Semen is routinely contaminated by extraneous flora as well as true pathogens. The presence of the enteric bacteria in semen is a definite clue to the contamination of the semen by fecal shedding while presence of pathogenic forms is rare and can lead to the conclusion that the bull is infected. The recent finding of an unusual pathogen like *Moraxella bovis* from semen is interesting because no other semen sample showed the presence of any other bacteria [15] which may be due to the added antibiotics in the semen extender and so the presence of this organism is somehow related to the establishment of a foci of infection by *Moraxella bovis* in the male genital tract from known or unknown sources. Injection of *Moraxella bovis* strain into pregnant mice, guinea pigs, rats, and rabbits however could induce abortion, death, embryo resorption, and production of small litters [16].

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