Wayamba Journal of Animal Science – ISSN: 2012-578X; P356 - P366, 2012
First Submitted July 25, 2012; Number 1344830870

ESTIMATION OF MICROBIAL LOAD AND THEIR BIOCHEMICAL PROPERTIES IN FROZEN SEMEN OF MURRAH BUFFALO BULLS *(BUBALUS BUBALIS)*

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Total 44 French mini straws (0.25ml) of frozen semen of Murrah buffalo bulls were collected from one of the frozen semen banks for evaluation of microbial load using the standard plate count (SPC) method using nutrient agar plate. These plates were incubated at 37^{o}C for 72 hrs and examined for growth. The average colony count was calculated and bacteria were also identified as Gram positive and Gram negative. A total of 9 biochemical tests were performed to characterize the isolates. Antibiotic sensitivity test was also performed to test the sensitivity against Ampicillin, Erythromycin, Gentamycin and Spectinomycin. The results indicate that nine samples out of 44 (20.45%) were found positive for various bacterial isolates. Most frozen semen doses except two bull Nos. MR-1017 and MBF-0338 were acceptable to use for Artificial Insemination as per the Bureau of Indian Standard (BIS). None of sample was found with fungal infection. Both the Gram positive and Gram negative bacteria were found in these samples. Results of biochemical properties of bacterial isolates are summarized. Each isolates are varying in their biochemical profile, but all the isolates exhibited negative urea hydrolysis test. The results of antibiotic sensitivity pattern in bacterial isolates were also summarized. All bacterial isolates exhibited variable pattern against Ampicillin, Erythromycin, Gentamycin and Spectinomycin. However, Bacterial isolate from bull number MBF-0338 showed inhibition to all antibiotics. Among all these antibiotics all bacterial isolates are resistance against Ampicillin. The concentrations of antibiotics were 10µg, 10µg, 10µg and 100 µg respectively. The article describes detailed investigation of microbial load in frozen semen of Murrah buffalo bulls.

Key words: Murrah buffalo, frozen semen, Microbial load, biochemical test, antibiotics

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Domestic buffalo occupy major place in Asia (97% of total world cattle population, as per Food & Agricultural Organization, 2000) and play a prominent role in rural livestock production by providing milk, meat and work draft force. Genetic improvement in these animals is carried out by worldwide accepted technique, Artificial Insemination (AI). This is achieved by using cryopreserved semen of potential buffalo bulls. Apart from providing desired genetic characteristics, the semen could acts as a vehicle for the wide distribution of undesirable pathogens (Vinodh et al., 2007). International trade of germplasm emphasizes the need for rapid, sensitive and specific diagnostic tests for certification of semen free from pathogenic agents. Bacterial isolation and co-culture methods are presently use to detect pathogens in the semen.

Artificial insemination (AI) was the first great technique applied to improve reproduction and genetics of farm animals, particularly in buffaloes, which seasonal breeders and show silent heat. In dairy industry, buffalo is considered an important milch animal in India because of higher fat and protein contents and as more than 60 percent of the milk produced is buffalo milk. Buffalo is considered to be a better converter

of fibrous feeds into milk, more resistant to disease and local climatic condition. The success of AI programme depends on quality semen production and processing in the lab. The bacterial contaminants of semen have been a major concern for most semen production laboratories as it adversely affects the semen quality (Diemer *et al*., 1996) and hence the subsequent fertility (Ochsendrof and Fuch, 1993, Griveau *et al*., 1995). Macrophages and polymorphonuclear granulocytes, which form the first line of defense against microorganisms, produce reactive oxygen species (ROS) to kill these microorganisms. ROS, however, is also released outside these cells and may react with the molecules and cells such as spermatozoa in their vicinity (Ochsendrof, 1998). The cellular antioxidants, present mostly in the cytoplasm, are scanty and inadequate to counteract this ROS because the sperm cell cytoplasm is very small (20 mm^3) and is mostly distributed on the midpiece (Drevius, 1970). The bacterial load in the semen samples is estimated by standard plate count (SPC) method. Few bacteria of semen survive at –196 ^oC in liquid nitrogen and acquire a certain level of resistance to antibiotics (Ronald and Prabhakar, 2001) and account for the contamination of approximately 50 % of frozen semen samples (Wierzbowski et al., 1984). The bacterial contaminants of the semen have been classified as pathogenic, potentially pathogenic or non-pathogenic. Gram's staining is very important method of differentiating bacterial species into two large groups; Gram positive and Gram negative, based on the chemical and physical properties of their cell wall (Gram 1884). Many semen banks in India are getting microbial load estimated in frozen semen doses by either identified laboratory or from outside agencies regularly (Hamaxi et al., 2011; Patel and Patel, 2012). Different biochemical tests are also used as additional tools to identify and characterize the bacterial isolates. In vitro sensitivity to different antibiotics is also performed by antibiotic sensitivity test so that suitable antibiotic in semen extender can be used. The present study was, therefore, conducted to study the microbial load (bacteria & fungi), their biochemical properties and antibiotic sensitivity of organisms present in the frozen semen of Murrah buffalo bulls. Therefore, this study was designed and planned to identify the bacteria in frozen semen of buffalo and also characterize them according to their morphological, cultural and biochemical properties. This study may be useful for evaluation of frozen semen at semen production station (semen bank).

MATERIALS AND METHODS

A total of 44 frozen semen doses from Murrah buffalo bulls were procured from one of the frozen semen banks in Gujarat. As per the standard practices followed by the semen laboratories, the semen was collected using sterilized artificial vagina, evaluated neat semen by various parameters, diluted in egg yolk tris glycerol dilutor for filling, sealing and freezing in French medium straws (0.25 ml). The microbial load was estimated using the standard plate count (SPC) method (Shukla, 2011) after incubating in nutrient agar plates by incubating at 37 ^0C for 24 h before inoculating with the semen sample from 10^{-1} and 10^{-2} dilutions. Two SPCA plates were taken for a single batch of semen and 0.1 and 0.5 ml semen sample was inoculated in each plate. These plates were incubated at 37 ^0C for 24 and 48 h before examination for the final results. The bacterial colonies were counted with the help of a colony counter. The average colony count was calculated. The semen was also examined for fungus, mucor and yeast by inoculating the samples in Sabaroud's dextrose agar at 37 ^0C for 24 h followed by subsequent examination of the organisms. Microorganism was also identified as Gram positive and Gram negatives. Antibiogram by using Ampicillin (A10) 10 mcg, Gentamycin (G10) 10 mcg, Tetracyclin (Ter30) 30 mcg, Erythromycin (E10) 10 mcg, Spectinomycin (Se100) 100 mcg were performed. Biochemical tests like methyl red test (Clarke and Kirner, 1941), Voges-Proskaur test (Voges and Proskauer, 1898), Citrate utilization test (Koser, 1924), Lead acetate paper strip test (Patnaik, 2002), Urea hydrolysis test (Rustigian and Stuart, 1941),

Nitrate reduction test (Conn and Breed, 1919), Indole test (Isenberg and Sundheim, 1958), Starch hydrolysis test (Skerman, 1967) and Triple sugar iron test (Sulkin and Willett, 1940) , were also performed to characterize the microorganism.

RESULTS AND DISCUSSION

The average microbial load from 44 frozen semen sample of buffalo bulls were analyzed by standard plate count agar (SPCA) method. Nine out of 44 samples were found positive for bacterial growth. The positive sample numbers for bacterial isolates are MR-1017, MBF-0338, MBF-368, MBF-398, MBF- 0392, MBF-0345, MBF-0326, MBF-0375 and MBF-0350 contain 1.50 x 10^3, 1.3 x 10^3, 1 x 10^2, 0.4 x 10^2, 0.8 x 10^2, 1 x 10^2, 2 x 10^2, 0.8 x 10^2 and 3 x 10^2 CFU/ml respectively (Table-1). Such frozen semen doses except bull Nos. MR-1017 and MBF-0338 were acceptable to use for Artificial Insemination as per the Bureau of Indian Standard (BIS). The BIS do not accept more than 500 CFU/dose. There were 4 different colonies observed in sample No. MBF-0338 were labeled as MBF-0338-(b), MBF-0338-(c), MBF-0338-(d), MBF-0338-(e) and also 4 different colonies observed in MBF-0392 were labeled as MBF-0392-(h), MBF-0392-(i), MBF-0392-(j), MBF-0392-(k), other seven samples shown only one but different colony were labeled as MR-1017-(a), MBF-0368-(f), MBF-0398-(g), MBF-0345-(l), MBF-0326-(m), MBF-0375-(n), MBF-0350-(o) (table-1 & figure-1). Also all the samples were analyzed using Sabouraud's dextrose agar (SDA) for detection of fungi. But all were shown negative results for fungi.

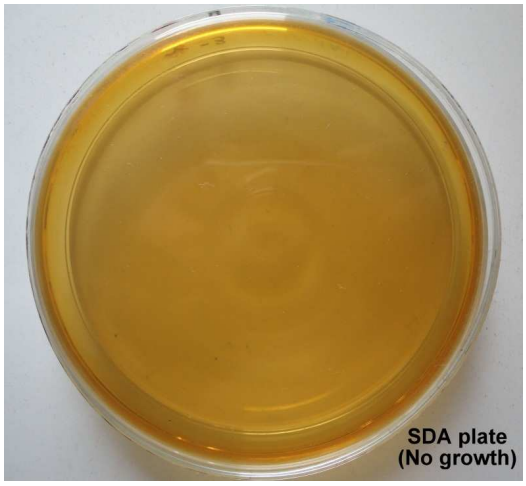


Fig.-1: Sabouraud's Dextrose Agar Plates indicating bactrial colony and no growth (control)

As mentioned above, 15 different bacterial counts ('a' to 'o') were observed in 9 samples of buffalo bulls. Their colony characteristics, gram staining, motility, acid-fast staining and endospore staining were summarized in table 2. Colony number MR-1017-(a), MBF-0338-(b), MBF-0338-(d), MBF-0338-(e), MBF-0398-(g), MBF-0345-(l), MBF-0326-(m), MBF-0375-(n), MBF-0350-(o) were gram positive organisms, and the colony number MBF-0338-(c), MBF-0368-(f), MBF-0392 (h), MBF-0392 (i), MBF-0392 (j), MBF-0392 (k) are gram negative organisms (table-2 & figure-2). All bacterial isolates were blue color, non-acid-fast and non-endospore former, except isolate number MBF-0350-(o) which was the only endospore former.

Bacterial contaminants in frozen semen sample were survived at -196^{o}C in liquid nitrogen and they acquired ascertain level of antibiotic resistant capacity (Ronald and Prabhakar, 2001). In this study only 9 out of 44 (20.45%) frozen semen samples were contaminated by bacterial contaminants. The higher level of bacterial contamination may indicate non-aseptic condition during semen collection and processing or those contaminants are resistant to antibiotic used in semen diluent liquid.

Table-1: Result of standard plate count (average colony forming unit per ml).

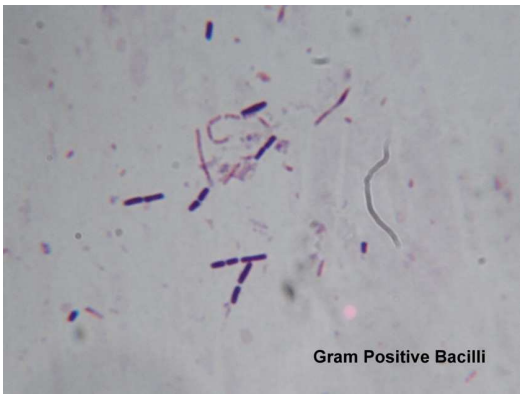
Sample No.	Dilution	Bacterial load (No. of colonies)	Average colony forming unit (CFU/ml)	Gram's nature	Fungus growth
MR-1017	10^{-1}	0.1 ml- 16 0.5 ml- 58	1.50×10^3	(a) Gram positive bacilli	No growth
	10^{-2}	0.1 ml- 2 0.5 ml- 7			
MBF-0338	10^{-1}	0.1 ml- 16 0.5 ml- 75	1.3×10^3	(b) Gram positivecocci (c) Gram negative cocobacilli (d) Gram positive cocci (e) Gram positive cocci	No growth
	10^{-2}	0.1 ml- 1 0.5 ml- 6			
MBF-0368	10^{-1}	0.1 ml- 1 0.5 ml- No growth	1×10^2	(f) Gram negative cocobacilli	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			
MBF-0398	10^{-1}	0.1 ml- No growth 0.5 ml- 2	0.4×10^2	(g) Gram positive bacilli	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			
MBF-0392	10^{-1}	0.1 ml- No growth 0.5 ml- 4	0.8×10^2	(h) Gram negative cocobacilli (i) Gram negative cocobacilli (j) Gram negative cocobacilli (k) Gram negative cocobacilli	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			
MBF-0345	10^{-1}	0.1 ml- 1 0.5 ml- No growth	1×10^2	(l) Gram positive bacilli	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			
MBF-0326	10^{-1}	0.1 ml- 2 0.5 ml- No growth	2×10^2	(m) Gram positive cocci	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			
MBF-0375	10^{-1}	0.1 ml- No growth 0.5 ml- 4	0.8×10^2	(n) Gram positive bacilli	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			
MBF-0350	10^{-1}	0.1 ml- 3 0.5 ml- No growth	3×10^2	(o) Gram positive bacilli	No growth
	10^{-2}	0.1 ml- No growth 0.5 ml- No growth			

Table-2: Colony characteristics and staining characteristics of bacterial isolates.

Characteristics	MR-1017-(a)	MBF-0338-(b)	MBF-0338-(c)	MBF-0338-(d)	MBF-0338-(e)
Size	Large	Large	Pinpoint	Medium	Medium
Shape	Irregular	Round	Round	Round	Round
Margin	Irregular	Entire	Entire	Entire	Entire
Elevation	Slightly raised	Slightly raised	Flat	Slightly raised	Flat
Texture	Rough	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque (concentric)	Opaque	Opaque	Opaque
Pigmentation	Off white	No pigment	Light yellow	No pigment	Off white
Gram's nature	Gram positive Bacilli	Gram positive cocci	Gram negative cocobacilli	Gram positive cocci	Gram positive cocci
Motility	Motile	Non motile	Motile	Motile	Motile
Acid-fastness	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast
Endospore	-	-		-	-

Characteristics	MBF-0368-(f)	MBF-0398-(g)	MBF-0392-(h)	MBF-0392-(i)	MBF-0392-(j)
Size	Small	Large	Medium	Large	Small
Shape	Oval	Round	Round	Irregular	Oval
Margin	Entire	Entire	Entire	Irregular	Entire
Elevation	Slightly raised	Flat	Flat	Flat	Slightly raised
Texture	Smooth	Smooth	Smooth	Smooth	Rough
Opacity	Opaque	Opaque	Opaque	Translucent	Opaque
Pigmentation	No pigment	No pigment	Off white	No pigment	No pigment
Gram's nature	Gram negative cocobacilli	Gram positive bacilli	Gram negative cocobacilli	Gram negative cocobacilli	Gram negative cocobacilli
Motility	Motile	Non motile	Motile	Motile	Non motile
Acid-fastness	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast
Endospore	-	-	-	-	-

Characteristics	MBF-0392-(k)	MBF-0345-(l)	MBF-0326-(m)	MBF-0375-(n)	MBF-0350-(o)
Size	Large	Large	Large	Large	Medium
Shape	Irregular	Irregular	Round	Irregular	Irregular
Margin	Irregular (concentric)	Irregular	Entire	Irregular	Irregular
Elevation	Flat	Raised	Flat	Flat	Submerged raised
Texture	Smooth	Smooth	Smooth	Smooth	Rough
Opacity	Transparent	Opaque	Opaque	Opaque	Opaque
Pigmentation	No pigment	No pigment	No pigment	No pigment	No pigment
Gram's nature	Gram negative cocobacilli	Gram positive Bacilli	Gram positive cocci	Gram positive Bacilli	Gram positive Bacilli
Motility	Motile	Non motile	Motile	Motile	Non-motile
Acid-fastness	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast	Blue color Non acid-fast	Blue color non acid-fast
Endospore	-	-	-	-	Endospore bearing



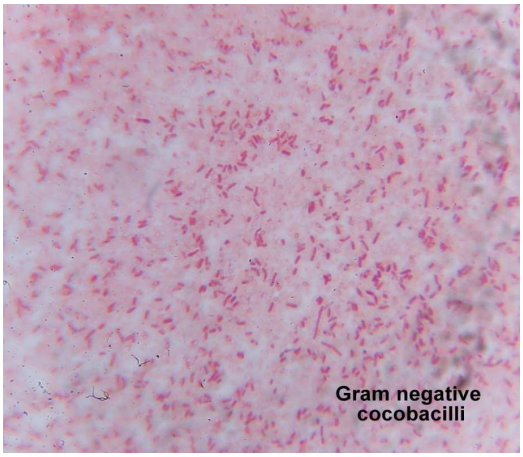
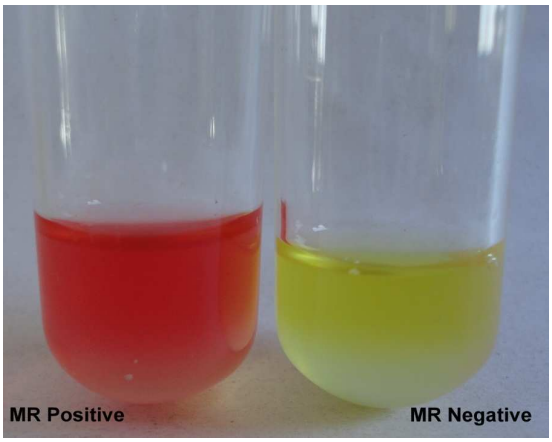


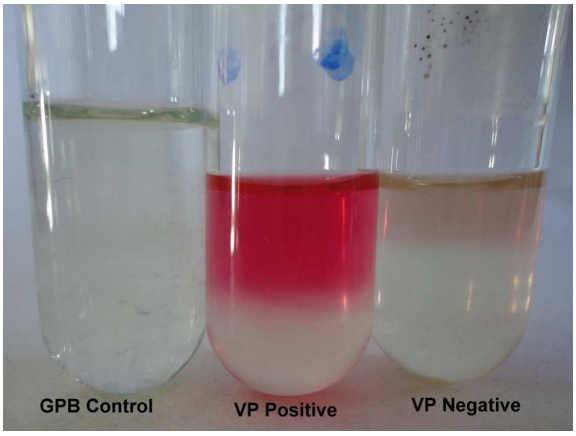
Fig-2: Gram staining showing Gram positive Bacilli and Gram Negative Cocobacilli

The results of biochemical properties of bacterial isolates were summarized in table-3. Each isolates showed their different biochemical properties. All the isolates exhibited negative urea hydrolysis test (Figure-3).

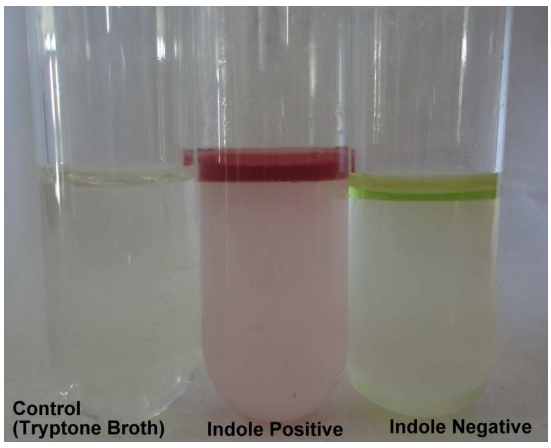
As per the database available in literatures (Aneja, 2011), the characteristics of isolates mentioned above are similar to the *Micrococcus lutes, Pseudomonas aeruginosa, Streptococcus lactis, Salmonella typhosa, Staphylococcus aureus, Proteus vulgaris* and *Enterobacteraerogens.* Some of isolates are similar to finding of Abro et al, (2009) where they isolated 7 pathogenic bacteria from frozen semen of cattle and identified.



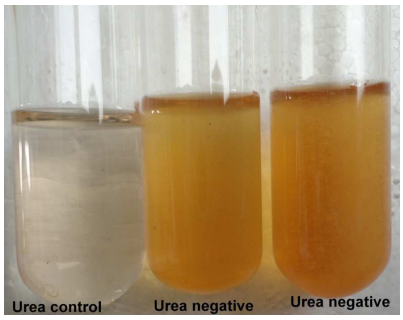
(A) Methyl Red test (GPB Broth)



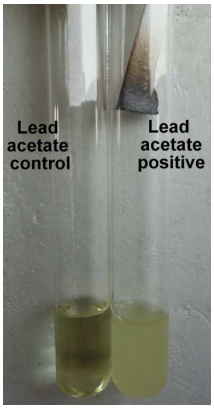
(B) Voges-Proskauer test (GPB Broth)



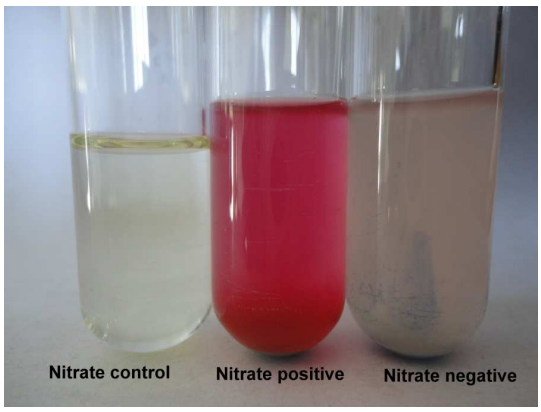
(C) Indole production test



(D) Urea hydrolysis test

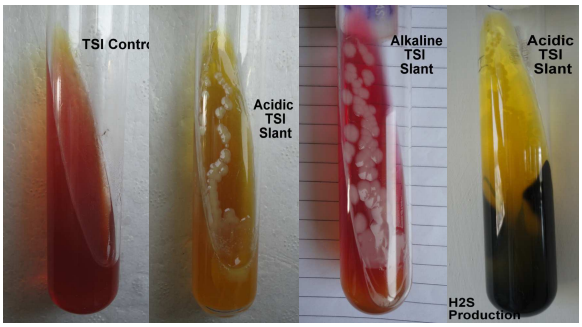


(E) Lead Acetate paper strip test (H_2S Production and Control & positive test)

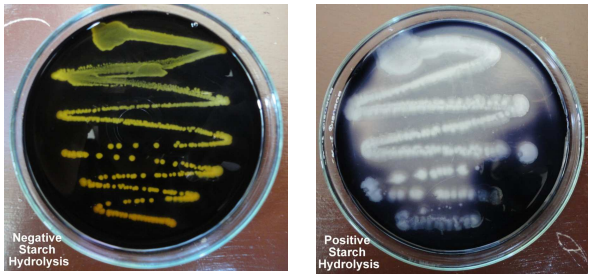


(F) Nitrate reduction test (peptone nitrate broth)

(G) Citrate utilization test (Simmons's Citrate Agar slant)



(H) Triple sugar iron test (TSI Slant) showing contol slant, acidic slant, H_2S production & alkaline slant respectively.



(I) Starch hydrolysis test (Positive & Negative)

Fig-3: Biochemical properties of bacterial isolates (A to I)

Antibiotic sensitivity test of all bacterial isolates were summarized in table 5 and figure-13. That was done by using agar disk method. All the bacterial isolates exhibited different sensitivity against Ampicillin (10µg), Erythromycin (10µg), Gentamycin (10µg) and Spectinomycin (100µg). Among all the antibiotics, all the isolates were found resistant againstAmpicillin. Bacterial isolate number MBF-0338 (d) showed inhibition to all antibiotics (table-4 and figure-4).

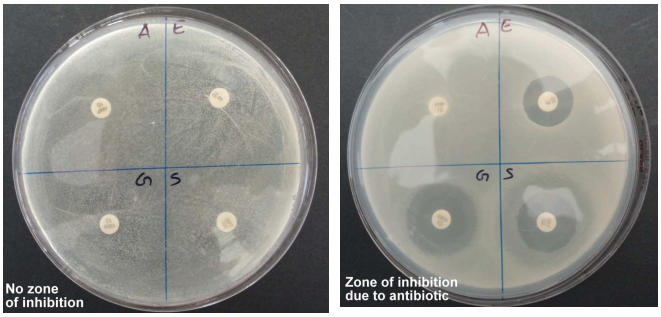


Fig-4: Antibiotic sensitivity test indicating Zone of inhibition, and No zone of inhibition

Table-3: Biochemical analysis of bacterial isolates.

Sample No.	MR	VP	I	UH	LA	NR	SH	CU	TSI
MR-1017-(a)	-	-	-	-	-	-	+	++	Acidic butt
MBF-0338-(b)	+	+	-	-	-	+	-	-	Acidic butt
MBF-0338-(c)	-	-	-	-	-	+	-	+	Acidic butt
MBF-0338-(d)	+	-	-	-	-	+	-	-	Acidic butt + slant
MBF-0338-(e)	+	-	-	-	-	+	+	-	-
MBF-0368-(f)	-	-	-	-	-	+	-	+	Alkaline slant
MBF-0398-(g)	+	-	-	-	-	+	+	+	-
MBF-0392-(h)	-	-	-	-	+	-	+	-	Alkaline slant
MBF-0392-(i)	+	-	-	-	+	-	+	-	Acidic slant, black butt (H_2S)
MBF-0392-(j)	-	+	-	-	+	+	-	-	Acidic slant
MBF-0392-(k)	+	-	+	-	+	+	-	-	Alkaline slant
MBF-0345-(l)	-	-	-	-	+	-	+	-	Alkaline slant, acidic butt
MBF-0326-(m)	+	-	-	-	-	-	+	-	Alkaline butt
MBF-0375-(n)	+	-	-	-	-	+	+	-	-
MBF-0350-(o)	+	-	-	-	-	-	+	-	Acidic slant

Where: **MR:** Methyl Red test; **VP:** Voges-Proskauer test; **I:**Indol production test; **UH:** Urea Hydrolysis test; **LA:** Lead Acetate paper test; **NR:** Nitrate Reduction test; **SH:** Starch Hydrolysis test; **CU:** Citrate Utilization test; **TSI:** Triple Sugar Iron test.

Table 4: Antibiotic Sensitivity tests of bacterial isolates.

S. No.	Sample no.	Zone of inhibition (in mm)			
		Ampicillin (A^{10})	Erythromycin (E^{10})	Gentamycin (G^{10})	Spectinomycin (Se^{100})
1.	MR-1017-(a)	No zone	10	19	22
2.	MBF-0338-(b)	No zone	21	20	23
3.	MBF-0338-(c)	No zone	23	24	19
4.	MBF-0338-(d)	No zone	No zone	16	No zone
5.	MBF-0338-(e)	No zone	No zone	No zone	No zone
6.	MBF-0368-(f)	No zone	30	30	20
7.	MBF-0398-(g)	No zone	21	20	20
8.	MBF-0392-(h)	No zone	11	15	No zone
9.	MBF-0392-(i)	No zone	11	20	20
10.	MBF-0392-(j)	No zone	10	17	11
11.	MBF-0392-(k)	No zone	17	18	17
12.	MBF-0345-(l)	No zone	No zone	22	23
13.	MBF-0326-(m)	No zone	30	16	20
14.	MBF-0375-(n)	No zone	17	22	30
15.	MBF-0350-(o)	No zone	17	16	28

Bacterial contamination in frozen semen first leads to the production of macrophages and polymorphonuclear granulocytes that is first line of defense against bacteria. Both the cells generate reactive oxygen species that in turn impair sperm function and reduces its fertilization capability (Oschendrof, 1998 and Morrell, 2006). Bacteria also adhere to spermatozoa and interfere with their motility (Bisson and Czyglick, 1974; Kaur et al, 1986 and Diemer et al, 1996). Microbes can also have direct reaction with acrosome or indirectly reacts by producing toxins (EI mulla, 1996, Morrell, 2006). It is documented that bacteria in the semen is controlled by using antibiotics in freezing diluents. Conventionally, benzyl penicillin and streptomycin sulphate alone or in combination is added at a concentration of a 1000 µg/ml respectively (Akhter et al, 2008). In the present study we investigated 4 antibiotics (Ampicillin, Erythromycin, Gentamycin and Spectinomycin) on all that fifteen bacterial isolates. Except Ampicillin, all antibiotics inhibited the growth of all bacterial isolates but their deleterious or toxic effect on spermatozoa to be evaluated before using them in cryopreservation of buffalo semen.

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