



Full Length Article

Biochemical Activities of Bacterial Species Isolated from the Frozen Semen of Cattle

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ABSTRACT

In order to study the bacterial contamination of frozen semen of cattle, 100 frozen semen samples collected from Semen Production Centers of Karachi and Rohri were analyzed. Biochemical and sugar fermentation tests were carried-out to confirm the specific characteristics of each bacterial isolate and to differentiate between various species of the same genus. Different species of bacteria isolated from the frozen semen samples were, *Acinetobacter*, *Actinobacillus lignieris*, *Citrobacter*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Staphylococcus intermedius*. *Actinobacillus lignieris* showed the positive to catalase, methyl red, methyl blue and urease, while negative to coagulase, methyl red, aesculin, Voges Proskauer and oxidase. *Acinetobacter* species exhibited high reactivity of catalase and urease enzymes. *Citrobacter* species showed negative reaction to indole and oxidase-negative and non-fermenters of mannitol, raffinose and lactose. *Micrococcus luteus* was showed variable reaction in Hugh and Leifson's medium and failed to degrade urea. *Pseudomonas aeruginosa* reacted positively to catalase, oxidase and gelatin liquefaction. While, negative for methyl blue, methyl red, Voges Proskauer, indole, hydrogen peroxide and hydrogen sulfide. *Staphylococcus intermedius* fermented mannitol, while *Staphylococcus epidermidis* did not. Gelatin was liquefied by *Staphylococcus intermedius*, while *Staphylococcus epidermidis* did not liquefy gelatin disc. *Staphylococcus epidermidis* was urease positive, while *Staphylococcus intermedius* was urease negative.

Key Words: Cattle frozen semen; Bacteria; Biochemical tests; Sugars fermentation

INTRODUCTION

Animal breeders have used artificial insemination (AI) extensively since the 1930s and 1940s, mainly in cattle and to some extent in pigs and sheep. By using the techniques available, semen can be collected from male animals at an artificial insemination centre and stored for varying periods at low temperatures. The semen can then be distributed to the surrounding area and throughout the country and can also be exported to other countries. Thus, as well as providing desired genetic characteristics, the semen could act as a vehicle for the wide distribution of un-desirable pathogens (Vinodh *et al.*, 2007). Increasing international trade in germplasm emphasizes the need for rapid, sensitive and specific diagnostic tests for certification of semen free from an increasing list of pathogenic agents (Afshar & Eaglesome, 1990; Eaglesome & Garcia, 1992; Eaglesome *et al.*, 1992). Bacterial isolation and co-culture methods are presently used to detect pathogens in the semen. Detection of infectious agents in the semen with a sensitive assay like

PCR would eliminate the use of infected animals as semen donors, thereby ensuring the reproductive health of inseminated cows. Endometritis, early embryonic death, repeat breeding and the consequence of which protracts the inter calving period, retained placenta and above all the most obvious manifestation mainly abortion can be thwarted. Similarly, a latently infected pedigreed bull need not be condemned for the fear of shedding the infectious agents, as a very sensitive method is now available for screening (Vinodh *et al.*, 2007). Therefore, this study was designed and planned to identify the bacteria in frozen semen of cattle and also characterize them according to their staining, morphological and biochemical characteristics.

MATERIALS AND METHODS

One hundred frozen semen samples were collected from the local semen production centres. The semen samples were taken in straws and sterilized bijou bottles in artificial insemination kits, which contained liquid nitrogen

and brought to the laboratory. New glassware was immersed in 1 N HCl solution and rinsed with 4 changes of normal tap water followed by 4 changes of distilled water and autoclaved. Oven dried Petri dishes were placed in a sterilizing box, while the small Petri dishes were wrapped in greaseproof brown paper. All the materials were then placed in a sterilizing oven at 165°C for 3 h. All the glassware with plastic caps or aluminum caps or all those could not resist dry heat in oven were sterilized in an autoclave at temperature of 121°C lb pressure for 15 min.

Dehydrated (Difco, 2000) nutrient agar, MacConkey agar and blood agar were rehydrated as recommended by (Difco, 2000) in distilled water. The media were stirred to dissolve and then autoclaved at 121°C under 15 lb pressure for 15 min. Cooled and blood agar at 45-50°C was added with 5% defibrinated aseptically sheep blood. The media were then dispensed into sterilized Petri dishes strictly under aseptic condition to avoid aerial and handling contamination as much as possible. The Petri dishes were left at room temperature overnight to check sterility. The samples were inoculated by streaking method on blood, nutrient and MacConkey's agar media and incubated aerobically at 37°C for 24 h for the growth of bacteria. These cultured bacteria were sub-cultured to obtain pure culture of bacteria under study. Simultaneously another single colony was picked up for the preparation of smear and stained with Gram's stain and Modified Ziehl Neelsen stain and examined under microscope for staining and morphological characteristics of the isolates. Further the colonies were picked-up for the pure culture for the biochemical characteristics and sugar fermentation tests. The different tests such as catalase; several bacterial colonies were picked, placed on a cleaned microscope slide and 2-3 drops of 3% of H₂O₂ solution in distilled water were added to the bacterial colonies on the slide. Immediate bubbling was noted as positive. Coagulase test; Two drops of distilled water were placed on cleaned microscope slide. A colony was picked up and mixed evenly to each drop of distilled water. A drop of rabbit plasma was then added to a drop of bacterial suspension, mixed and rocked gently. Formation of bacterial cell clumps in plasma containing drop recorded as positive reaction. Gelatin liquefaction; Peptone water medium was incubated with a bulk of colonies of bacteria, a charcoal gelatin disc (Oxoid, 2000) gently with peptone water aseptically placed in the culture medium and incubated at 24-77 h at 37°C. A positive reaction indicates the release of carbon particles into the medium with eventual complete disintegration of the charcoal disc. The disc remained intact in case of negative reaction. Aesculin test; aesculin agar was sterilized in an autoclave at temperature of 121°C lb pressure for 15 min. Cooled to 45-50°C and then aseptically 20 mL serum was added. Mixed well and dispensed into universal bottles, solidified in slanting position and then incubated at 37°C for 48 h for sterility checking. Aesculin agar slant was cultured with the bacterium, incubated at 37°C for 24-48 h. A positive aesculin hydrolysis black

precipitation. Bile tolerance test; ox bile agar was autoclaved at temperature of 121°C lb pressure for 15 min. Cooled to 45-50°C and then aseptically horse serum was added aseptically. Dispensed in 5 mL volume in Bijou bottles, then these were inoculated with isolated bacterial colonies and incubated up to 48 h at 37°C. The growth on the medium indicated bile tolerance. Hugh and Leifson's test; medium was autoclaved at temperature of 121°C lb pressure for 15 min and poured in 10 mL universal bottles. The medium was sown by stabbing with straight wire, some of them were sealed to exclude oxygen. The indicator bromothymol blue, which is green at neutral pH, yellow at acid and blue in alkaline pH. Indole production test; bacteria were cultured in peptone water and incubated at 37°C for 24 h. About 0.5 mL of culture was removed into a narrow 5 mL test tube and 0.2 mL of Kovacs reagent was added to it, shaken gently and allowed to stand for 5 min. A positive test is noted with a red coloration in the reagent layer. Methyl red and Methyl blue; 5 mL of dextrose phosphate broth were cultured in Bijou bottles to 24 h, added with methylene red and methylene blue indicators and incubated at 37°C for 1-3 h and results were noted by change of color. Voges Proskauer test oxidase; 5 mL from 48 h old culture of the bacterium in dextrose phosphate broth were pipetted into small test tubes and 3 mL of 5% alpha-naphthol in methyl alcohol were added. 1 mL of 40% solution of KOH was added to tube, sealed with paraffin sealer, shaken well at intervals and results were noted bright pink indicated positive. TSI; TSI slants were inoculated with isolated organisms and incubated at 37°C for 24 h and the results were noted up to 72 h of incubation. Simmon's citrate; Simmon's citrate agar was inoculated with bacteria and incubated at 37°C for 72 h. It was examined after every 24 h and color change. Urease production; Christensen urea medium (Christensen *et al.*, 2002) was inoculated with organisms and incubated at 37°C for 72 h, and positive reaction was noted by change in color. Nitrate reduction; nitrate blood agar was autoclaved at temperature of 121°C lb pressure for 15 min and cooled to 50°C. Then 1 mL of 10% KNO₃ solution was added to blood agar base, mixed thoroughly and 5 mL of aseptically collected defibrinated horse blood was added and dispensed into Petri dishes. Dry blood agar plate was stab inoculated and incubated overnight at 37°C. Sugar fermentation test; the oxidation fermentation medium was dispensed in Bijou bottles. The sugar to be tested was added at the rate of 0.2 mL of 10% solution. Bijou bottles with sugar containing medium were inoculated with organisms. One of these was overlaid with a layer of liquid paraffin and then both were used for determination of sugar fermentation of each sugar, as prescribed by Khalil and Gabbar (1992).

RESULTS

The present investigation demonstrated that out of hundred samples of frozen semen of cattle examined, 7 were

found positive for various bacterial isolates, while 93 were negative without any bacterial growth. The frozen semen samples of cattle were contaminated with *Acinetobacter*, *Actinobacillus ligneirisi*, *Citrobacter*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Staphylococcus intermedius*.

The biochemical and sugar fermentation properties of different bacterial organisms identified from the frozen semen of cattle, which helped to separate them from each other. Because they are very close in their characteristics and difficult to distinguish name them individually.

Acinetobacter. The species reacted positively to catalase, methyl blue, urease and indole. They did not show negative to coagulase, methyl red, aesculin, Voges Proskauer and oxidase. It did not produce hydrogen sulfide gas and acted triple sugar iron (TSI) and produced K/K (alkaline slant/alkaline butt). It failed to reduce nitrates to nitrites. It utilized Simmon's citrate and hydrolyzed gelatin disc. It was oxidative on Hugh and Leifson's medium and produced alkaline reaction. The microorganisms of this species fermented glucose, maltose, lactose, rhamnose, sucrose, xylose and dulcitol and produced acid and gas, while did not effect inositol, mannitol, raffinose, arabinose and galactose. The results found in the investigations are presented in Tables I and II.

Actinobacillus ligneirisi. The positive results were recorded for catalase, methyl red, methyl blue and urease. It was negatively reacted to indole, hydrogen peroxide, gelatin liquefaction, aesculin, Voges Proskauer and oxidase. It reduced nitrates to nitrites. It showed A/A (Acidic slant/Acidic butt) in TSI sugar and produced hydrogen sulfide gas. It was oxidative on Hugh and Leifson's medium. The microorganisms of this species utilized glucose, mannitol, maltose, lactose, raffinose, arabinose, sucrose, xylose and galactose. and produced acid, while did not effect dulcitol, inositol and rhamnose (Table I & II).

Citrobacter. *Citrobacter* species was observed as catalase, oxidase, methyl red, methyl blue, Voges Proskauer and hydrogen sulfide positive while negative for indole, coagulase, hydrogen peroxide, aesculin and urease. It produced K/A (alkaline slant/acidic butt) in TSI sugar medium. It reduced nitrates to nitrites and liquefied gelatin disc. It utilized Simmon's citrate. The microorganisms of this species fermented glucose, inositol, maltose, rhamnose, sucrose and produced un-known gas from the glucose, while did not ferment mannitol, raffinose, lactose, arabinose, xylose, dulcitol and galactose (Table I & II).

Micrococcus luteus. *Micrococcus luteus* was biochemically observed as positive to catalase, oxidase, methyl blue, Simmon's citrate, gelatin liquefaction and aesculin, while negative to indole, coagulase, methyl red, hydrogen peroxide and Voges Proskauer. The organism showed variable reaction in Hugh and Leifson's medium. It failed to degrade urea. It utilized TSI sugar K/A (alkaline slant/acidic butt). The cells of this species fermented glucose, mannitol,

maltose, xylose, dulcitol and galactose, while did not ferment inositol, rhamnose, raffinose, lactose, sucrose and arabinose (Table I & II).

Pseudomonas aeruginosa. The species reacted positively to catalase, oxidase and gelatin liquefaction. The organism was negative for methyl blue, methyl red, Voges Proskauer, indole, hydrogen peroxide and hydrogen sulfide. It slowly hydrolyzed urea and failed to reduce nitrates to nitrites. It utilized Simmon's citrate. However, it acted on TSI and changed medium in K/K (alkaline slant/alkaline butt). On Hugh and Leifson's medium *Pseudomonas aeruginosa* produced acid only in the open tube, since its breakdown of the carbohydrates was oxidative. The acid was formed first in the upper part of medium, which was near to oxygen and also produced pigments. Furthermore, *Pseudomonas aeruginosa* produced acid and gas from glucose, mannitol, raffinose, xylose and galactose. It did not act on the dulcitol, inositol, lactose, maltose, rhamnose, sucrose and arabinose (Table I & II).

Staphylococcus epidermidis. The positive results were recorded for catalase, methyl blue, hydrogen sulfide and urease.

It gave negative reaction to oxidase, gelatin liquefaction and indole, coagulase, methyl red, hydrogen peroxide and Voges Proskauer. It reduced to nitrates to nitrites and did not utilize Simmon's citrate. In TSI sugar medium, it reacted A/A (Acidic slant/Acidic butt) (Table I & II).

Staphylococcus intermedius. The catalase, methyl blue, gelatin liquefaction and nitrate reduction were observed positive for *Staphylococcus intermedius*, while coagulase, oxidase, methyl red, indole, hydrogen peroxide and Voges Proskauer were recorded negative. It did not hydrolyze urea. It produced A/A (Acidic slant/Acidic butt) and produced hydrogen sulfide in TSI sugar medium. It fermented glucose, mannitol, lactose, maltose, raffinose, sucrose, xylose and dulcitol and galactose and did not ferment inositol, rhamnose, arabinose and galactose.

DISCUSSION

There increase activity of catalase and urease enzymes exhibited by cells of *Acinetobacter* species, may be due to these enzymes have profound effect on the breakdown of hydrogen sulfide bond present in cell wall of the cells. There was no reactivity of oxidase, this can be due to presence of strong hydrogen bonds. The cells grown on methanol exhibited higher catalase activity than those grown on pyruvate or glucose and also showed peroxidase activity. The biochemical changes produced in different substances of various chemical compositions by *Acinetobacter* species are same as mentioned by Eugene *et al.* (1997) and Collins and Patricia (1999) in their study. On other hand, Khalil and Gabbar (1992) stated some what different results partially do agree to findings presented by above authors.

Table I. Biochemical properties of bacterial species isolated from the frozen semen of cattle

Bacterial species	Triple sugar	Coagu Iron	Oxid lase	Catal ase	Methyl blue	Methy red	Simmon citrate	Voges Proskauer	Ind ole	Hydrogen peroxide	Gelatin liquefaction	Hydrogen sulfide	Urea hydrolysis	Hugh and Leifson	Nitrate reduction	Aeus clin
<i>Acinetobacter</i>	K/K	-	-	+	+	-	+	-	+	+	+	+	+	+	+	-
<i>Actinobacillus Ligneirisi</i>	A/A	-	-	+	+	+	+	-	-	-	-	+	+	+	+	-
<i>Citrobacter</i>	K/A	-	+	+	+	+	+	+	-	-	+	+	-	-	+	-
<i>Micrococcus luteus</i>	K/A	+	+	+	+	-	+	-	-	-	+	-	-	-	-	+
<i>Pseudomonas aeruginosa</i>	K/K	-	+	+	-	-	+	-	-	-	+	-	+	-	-	-
<i>Staphylococcus epidermidis</i>	A/A	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-
<i>Staphylococcus intermedius</i>	A/A	-	-	+	+	-	-	-	-	-	+	+	-	-	+	-

Table II. Sugar fermentation of bacterial species isolated from the frozen semen of cattle.

Bacterial species	Glucose	Insitol	Lactose	Mannitol	Maltose	Raffinose	Rhamnase	Sucrose	Arabinose	Xylose	Glactose	Dulicitol
<i>Acinetobacter</i>	+	-	+	-	+	-	+	+	-	+	-	+
<i>Actinobacillus Ligneirisi</i>	+	-	+	+	+	+	-	+	+	+	+	-
<i>Citrobacter</i>	+	+	-	+	+	-	+	+	-	-	+	-
<i>Micrococcus luteus</i>	+	-	-	+	+	-	-	-	-	+	+	+
<i>Pseudomonas aeruginosa</i>	+	-	-	+	-	+	-	-	-	+	+	-
<i>Staphylococcus epidermidis</i>	+	-	+	-	+	-	-	+	-	+	-	-
<i>Staphylococcus intermedius</i>	+	-	+	+	+	+	-	+	-	+	-	+

Citrobacter species are mostly carbon dependent as utilize carbon as a sole source of energy and showed negative to indole. The findings of present study contracted with Collins and Patricia (1999) described species were indole variable. The bacterial species were found to be oxidase-negative and non-fermenters of mannitol, raffinose, lactose, arabinose, xylose, dulcitol and glactose. It was observed from negative oxidase and non-fermenting ability due to inhibition of release of gases during reactivity of enzymes. Kitch *et al.* (1994) found that *Citrobacter* species were oxidase-negative, gram-negative nonfermenters. However, the findings of Khalil and Gabbar (1992) and Khan (2000) recorded almost similar findings regarding the biochemical properties and fermentation of sugars, which were recorded in this study.

It was observed that *Micrococcus luteus* was differs from other species of *Micrococcus* in hydrolysis of glucose, mannitol, maltose and xylose. In some respects catalase, methyl blue, Simmon's citrate and gelatin liquefaction. Liu *et al.* (2000) investigated that *Micrococcus luteus* was positive to urease and methyl red, while negative to indole formation, nitrate reduction and Voges Proskauer. All biochemical and sugar fermentation tests carried out on *Micrococcus luteus* for identification in different chemicals during present survey are the same recorded Shaikh (1999) and Nizamani (1999) obtained similar results in their investigations.

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. The production of acid and gases from glucose, mannitol, raffinose, xylose and glactose was also noted by action of oxidase reaction. The biochemical properties of the organism recorded in this study are the same as obtained by Sharma and Adlakha (1996); Nizamani (1999) and Parkash (2000). But Collins and Patricia (1999) reported some variable results; therefore our findings are partially agreed to observations made by Collins and Patricia (1999).

The biochemical reactions of *Staphylococcus epidermidis* and *Staphylococcus intermedius* indicated that both organisms were highly sensitive to catalase and nitrate reduction reactions. *Staphylococcus intermedius* fermented mannitol possible reason to free hydroxyl group to membrane bound protiens, while *Staphylococcus epidermidis* have lack of these protiens, which oxidise the mennitol. Nilsdotter *et al.* (2005) reported that the adherence of *Staphylococcus epidermidis* to extracellular matrix proteins and effects of fibrinogen-bound bacteria on oxidase activity and apoptosis in neutrophils. Beatriz *et al.* (2006) in their investigations observed that a high number of coagulase negative *Staphylococcus* strains were also enterotoxin producers. All the proerties of *Staphylococcus epidermidis* are accordance to the findings established by Nizamani (1999), Merchant and Packer (1999) and Parkash (2000). The properties tested during biochemical investigations, all changes were the same and no any distinguishable changes were observed.

CONCLUSION

It has been concluded from this study that the isolates can be biochemically distinguished from the known species and confirm the specific characteristics of each bacterial isolate and also to differentiate between various species of the same genus. 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.

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