JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 561-571

A Study on Isolation and Characterization of *Pasteurella multocida* for Clinically Positive Cases of Hemorrhagic Septicemia in Buffaloes

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(Received: 15 September 2014; accepted: 07 October 2014)

Haemorrhagic septicaemia (HS) is one of the most common bacterial disease of buffaloes, cattle, sheep and goat. Buffaloes are more susceptible to it than are cattle. The causative organism (*Pasteurella multocida*) was isolated from clinically positive cases of hemorrhagic septicemia and identified from blood samples based on morphological characteristics. The organism with Gram's staining method appeared Gram negative under oil immersion lens, as coccobacillary thin rods with rounded ends and non-spore forming cells. The size of the organism was variable with repeated subculturing but the shape remained almost consistent with a tendency to bipolar staining. The capsule of the organism was observed as delineated with India ink and bipolarity was observed as dumble shaped. The sugar fermentation was uniform for all the suspected isolates induced fermentation of glucose, sucrose, mannose and fructose with the production of acid only and no gas. Pathogenicity of *P. multocida* was confirmed in rabbits. It proved highly virulent to rabbits and they died within 24 hours. No death was recorded in control. The postmortem lesion observed included those of generalized septicemia like congestion of the internal organs, excessive hemorrhage particularly on laryngeotracheal region, accumulation of inflammatory fluid in the thoracic and peritoneal cavities.

Key words: *Pasteurella multocida*, Hemorrhagic septicemia, LPS, ELISA, Immunity & Buffaloes.

The Gram-negative bacterium *Pasteurella multocida* exhibits a broad host range including most mammals, birds and also humans (Adlam and Rutter, 1989; Quinn *et al*., 1994). Pasteurellosis is one of the most significant bacterial diseases of rabbits and one of the major causes of considerable economic loss in large production units throughout the world (Manning, 1982; Takashima *et al*., 2001). The disease is characterised by various clinical syndromes, e.g. respiratory distress, genital affections, abscesses and septicemia, but infection by *P. multocida* can also appear without any clinical signs manifested (DiGiacomo *et al*., 1983; Delong and Manning, 1994). The variability in clinical signs as well as the course of the disease may be influenced by different *P. multocida* virulence factors such as a capsule, fimbriae, lipopolysaccharides (endotoxin), dermonecrotoxin, neuraminidase etc. (DiGiacomo *et al*., 1989; Straus *et al*., 1996). Capsular typing of *P. multocida* rabbit

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isolates showed that to date, pasteurellosis in rabbits is mainly caused by the capsular type A and, to a lesser extent, capsular type D strains (Kawamoto *et al*., 1990; Vandyck *et al*., 1995; Dabo *et al*., 1999).

A number of subcellular fractions have been tested for the induction of immunity in experimental animals. These fractions are: a ribosomal fraction (Baba, 1977), purified or cloned outer membrane proteins (Abdullahi *et al*., 1990; Adler et al., 1999), a ribosomal lipopolysaccharide (LPS) complex (Philip and Rimler, 1984), purified LPS (Rebers *et al*., 1980), LPS-protein complexes (Rebers and Heddleston, 1976) and potassium thiocyanate (KSCN) extracts (Mukkur, 1978; Ryu, Kaeberle, 1986). Many of these subcellular fractions provided a degree of protection in experimental animals and the role of humoral immunity against *P. multocida* infection has been suggested. However, it is not certain whether protection against *P. multocida* infection involves cellular immunity. This study was aimed is the isolation and characterization of *Pasteurella multocida* for clinically positive cases of Hemorrhagic septicemia in buffaloes.

MATERIALS AND METHODS

The Infected field samples were collected from Landhi, Cattle Colony. The animals showing typical signs and symptoms of the disease were identified. The blood from the jugular vein and nasal swabs was collected from live animals while the heart blood was collected from dead animal during necropsy. The samples were transported on ice. These samples were cultured on the nutrient agar, CSY agar, blood agar and Mc-Conkey's agar for isolation and identification of the causative organism.

Isolation of organism from samples

On arrival at the laboratory, the isolation of the organism was done by culturing the samples of plasma on blood agar and Tryptose yeast extract agar and overnight incubation was done at 37°C to study the culture characteristics.

Slide smears were prepared from the selected colonies, fixed and stained by Gram's staining technique to observe the morphology microscopic (Buxton and Fraser, 1977). For the demonstration of the capsule, the smear was stained with wet India ink staining method (Collee *et al*., 1989). For bipolar staining (Cruickshank, 1968) smear was stained with methylene blue for 3 minutes. Then washed with water. These slides were examined under microscope with an oil immersion objective.

After confirming with different staining techniques, the selected colonies were cultured on Tryptose yeast extract agar and MacConkey agar and following biochemical tests were performed for Robert Typing *P. multocida* (Collee *et al*., 1989). Sugar fermentation reactions with arabinose, xylose, glucose, fructose, sucrose, lactose, mannitol and inositol were performed. The biochemical tests such as Indole production test, Methyl red test, Nitrate reduction test, Hydrogen sulfide production test, Catalase test and Gelatin liquification test were conducted as described by Buxton and Fraser (1977). After confirming with cultural and biochemical tests, the selected colonies were cultured on CSY agar for further analysis.

Pathogenicity Test

24 hours growth from Tryptose Yeast extract agar slant was suspended in 1 ml sterile physiological saline solution as described by Bain *et al.*, 1982. The mean suspension density of each sample having 1×10^9 CFU/ml was evaluated at 540 nm. To test the pathogenicity of samples, mice were selected and 0.5 ml growth suspension was injected intraperitoneally.

Postmortem examination of the dead animals was performed and re-isolation of the organism from the heart blood were cultured on Tryptose Yeast extract agar and incubated at 37 °C for 24 hours. Standard method of media preparation and biochemical test described by Buxton and Fraser (1977), Wijewardana (1992) and Cruickshank (1968) were adopted.

Serological test for capsular typing

Preparation of antisera

The antiserum against reference strain *P. multocida* Carter type B was prepared. Antisera were developed in four adult rabbits. Animals were inoculated twice intravenously at four-day intervals with 0.5ml, 1ml, 1.5ml and 2ml of formalin inactivated culture. The mean suspension density of each sample having $1x10^9$ CFU/ml was evaluated at 540 nm. Animals were kept for seven days after final inoculation. No preservative was added to the collected antisera. The antisera had a titer of 1: 512.

Indirect haemagglutination test

Broth culture of 6-8 hours old of a reference strain was streaked on to CSY agar plates and 24 hours incubation was done at 37 °C. The growth was harvested in 3 ml physiological saline (0.3 % formalin) was used for harvesting the growth. After heating this suspension at 56 °C for half an hour, then centrifugation was done and the clear supernatant fluid was used as antigen extract. The same methodology was adopted for the preparation of antigen extracts from unknown strains that was to be typed.

Aseptically collected sheep blood with an anticoagulant (EDTA) was centrifuged. Packed RBC's were given three washings in sterile saline. The antigen extracted from an unknown strain was used for these RBCs. Mixed antigen extract with packed RBCs (15: 1) and then incubated this mixture for 1 hour at 37 °C with repeated shaking did the sensitization. The sensitized RBCs were recovered by centrifugation, washed thrice-in sterile physiological saline and made up to a final 1 % suspension in physiological saline. The type specific hyper-immune antiserum was absorbed by the addition of packed RBCs (3:1) for 30 minutes at room temperature, and then centrifuged at 500 *g* for 10 minutes to pellet the RBCs. The absorbed antiserum was then inactivated by heating at the 56 OC for 30 minutes.

Test procedure

For IHA two fold serial dilution of test sera (100µl) were prepared up to column, 11th while the 12th column was used as control having standard sera in four wells and four wells contained only normal saline.Sensitized RBCs with 100µl amount were added in all the wells. Microtitration plates were then agitated and incubated at 37^{o} C for 15 to 30 minutes. The results were noted at 15 minutes and 30 minutes after incubation and were compared with each other. The button formation of RBCs indicated negative test, whereas lumped RBCs indicated positive test as described by Carter (1955).

Statistical analysis

Analysis of variance (ANOVA) was used under Duncan's multiple range test to compare the antibody titer between different groups at different days (Steel and Torrie, 1984).

RESULTS AND DISCUSSIONS

Culture characteristics

All the samples were examined for their culture characteristics on various media and observations recorded after 24 hours aerobic incubation at 37 ºC. *P. multocida* is a facultative anaerobic bacterium that grows best at 37 ºC. The isolates that exhibited luxuriant growth, having off white mucoid and sticky colonies of a large size being 2 mm in diameter and also not producing haemolysis on blood agar were subjected to staining and biochemical reactions for isolation of typical *P. multocida*. Such bacterial growth was observed in 27 out of 40 samples. While in case of TYE agar the colonies showing round, sticky mucoid consistency slightly elevated in the center about 2-3mm in diameter were selected for staining and biochemical reactions for the isolation and confirmation of *P. multocida* (Fig. 1).

The organism with Gram's staining method appeared Gram negative under oil immersion lens, as coccobacillary thin rods with rounded ends and non-spore forming cells. The size of the organism was variable with repeated subculturing but the shape remained almost consistent with a tendency to bipolar staining. The capsule of the organism was observed as delineated with India ink and bipolarity was also observed as dumble shaped. The suspected 27 samples out of 40 samples showing characteristics of *P. multocida* were selected and cultured on CSY agar to purify the culture. Results of morphological and cultural characteristics shown in Table 1 and Fig. 2.

Biochemical tests

Sugar fermentation test

The sugar fermentation was uniform for all the suspected isolates induced fermentation of glucose, sucrose, mannose and fructose with the production of acid only and no gas. The sugar fermentation results of all the isolates are presented in Table 2 and Fig. 3. All the suspected isolates showed a uniform biochemical reaction similar to sugar fermentation reaction, for which they were tested. A positive reaction for catalase test, indole production test, nitrate reduction test and hydrogen sulfide production test and showed a negative reaction to methyl red test, gelatin

Table1. Results of Morphology and Cultural Characteristics of field isolates collected from clinically positive cases of HS (1-40)

S.	Morphology and staining			Cultural Characteristics			
No.	Staining Reaction	Capsule	Bipolarity	Blood agar	TYE agar	McConkey's agar	CSY agar
1.	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
2	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
3	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish round colonies	Rounded sticky colonies
4	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
5	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
6	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish round colonies	Rounded sticky colonies
7	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
8	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
9	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
10	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
11.	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
12	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size

13	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
14	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
15	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish round colonies	Rounded sticky colonies
16	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
17	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
18	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
19	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
20	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish round colonies	Rounded sticky colonies
21	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
22	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
23	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
24	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
25	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
26	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size

27	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
28.	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish round colonies	Rounded sticky colonies
29	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
30	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
31	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
32	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
33	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
34	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
35	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish round colonies	Rounded sticky colonies
36	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
37	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
38.	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size
39	Gram –ve, Small bacilli,	Absent	Absent	Haemolysis showed	Small colonies	Small whitish colonies	Rounded sticky colonies
40	Gram –ve, Small coco bacilli, thin rods with rounded ends	Present	Dumble shape	Luxuriant growth without haemolysis	Smooth, slightly raised in the center, mucoid and sticky in nature	No growth	Smooth, yellowish, mucoid and larger in size

Table 2. Results of Sugar fermentation, Biochemical, Pathogenicity tests & serotyping of field "isolates" collected from clinically positive cases of HS (1-40) "isolates" (1-40)

Sample No.	Sugar fermentation tests						Biochemical tests						Pathogenecity test	Sero typing	
	Glucose	Lactose	Maltose	Sucrose	Salicin	Mannose	Fructose	H_2S Production test	Methyl red test	Gelatin liquification test	Indole production test	Nitrate reduction test	Urease test	Catalase test	Mice inoculation test	IHA titer with reference serum (Type B)	Inference
1.	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	1: 256	*P. multocida* (B)
2.	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	1:512	*P. multocida* (B)
3.	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-	Unknown
4.	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	1: 256	*P. multocida* (B)
5.	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	1: 256	*P. multocida* (B)
6.	-	-	+	+	-	+	+	+	+	-	-	+	+	+	-	-	Unknown
7.	+	-	+	+	-	+	+	-	-	-	+	-	-	+	-	-	Unknown
8.	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	1:512	*P. multocida* (B)
9.	-	-	-	+	-	-	+	+	-	-	-	+	+	+	-	-	Unknown
10.	+	-	+	+	-	+	+	+	-	-	+	+	-	+	+	1: 256	*P. multocida* (B)
11.	+	-	-	+	-	-	+	-	+	+	+	-	-	+	-	-	Unknown
12.	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	1:512	*P. multocida* (B)
13.	+	+	-	+	-	+	+	+	-	-	+	+	-	+	+	1: 256	*P. multocida* (B)

14.	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
15.	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	Unknown
16.	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	Unknown
17.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1: 256	+	*P. multocida* (B)
18.	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
19.	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
20.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Unknown
21.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
22.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
23.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
24.	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	Unknown
25.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
26.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
27.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
28.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	Unknown
29.	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
30.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
31.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
32.	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	Unknown
33.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
34.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
35.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	Unknown
36.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
37.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)
38.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:256	+	*P. multocida* (B)
39.	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	Unknown
40.	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	1:512	+	*P. multocida* (B)

liquefaction test and doesn't show areas activity on urea agar plates of showing typical signs of *P. multocida* while the other 13 samples showed variable results not relating to *P. multocida* (Table 2).

Pathogenicity

Pathogenicity of *P. multocida* was confirmed in rabbits. It proved highly virulent to rabbits and they died within 24 hours. No death was recorded in control. The organism was re-isolated from the heart blood of each dead animal and its identity was reconfirmed The postmortem lesion observed included those of generalized septicemia like congestion of the internal organs, excessive hemorrhage particularly on laryngeotracheal region, accumulation of inflammatory fluid in the thoracic and peritoneal cavities (Table 2).

Fig. 1. Growth of *Pasteurella multocida* on CSY and Nutrient agar

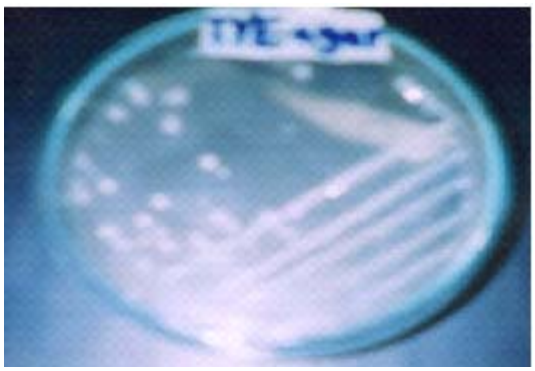

Fig. 2. Growth of *Pasteurella multocida* on TYE agar

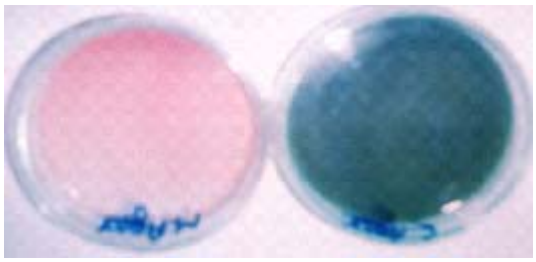

Fig. 3. No Growth of *Pasteurella multocida* on Solid media, MacConkey's agar (Left Plate) and Citrate agar (Right Plate)

Serotyping

There are numerous classification and typing techniques of *P. multocida.* In this study serotyping techniques of Carter (1955) were followed. Results of IHA confirmed that only Carter type B (Robert type-I) is associated with the disease (Table 2).

The organism was identified based on morphology, staining reactions and culture characteristics, on microscopic examination; the organism appeared as coccobacillus, capsulated, gram negative and stained bipolar. With repeated sub-culturing the organism tends to diminish in size and became somewhat rounded and sometimes even lost its bipolar character. These findings were in complete agreement with those reported by Topely (1998) and Kumar *et al.* (2004). In wet India ink staining method, capsule appeared as white lined with India ink as also studied by Bain *et al.* (1982).

All the isolates fermented the glucose. This had also been reported by Aslam *et al.* (1988) and Mohan *et al*. (1994). Lactose was only fermented by isolating No.16 while all other isolates did not ferment Lactose. This shows that lactose gave variable results as reported by Kozarev and Mamadudian (1988) that variability was found in patterns of fermentation of levulose, arabinose, xylose, maltose, lactose and raphnose. In case of sucrose all, the isolates were able to ferment it. These results are similar to Aslam *et al.* (1988) and Mohan *et al.* (1994) who reported that consistent results were obtained in the test for glucose, inositol, salicin and sucrose. There was an obvious relationship between serotype, host or disease and their pattern of utilization of certain substrates by an isolate. The mannose was also fermented by all the isolates, these results were uniform. The similar results were obtained by Bain *et al*. (1982). Salicin was not fermented by any of isolate. These results were in agreement with Bain *et al*. (1982). Mohan *et al.* (1994) also reported that salicin was not fermented by *P. multocida*. Fermentation of maltose was variable in our experiment; two isolates 10 and 20 did ferment the maltose. While

all other isolates did not ferment the maltose, which is also discussed by Kozarev and Mamadudian (1988).

All the isolates were positive for indole production test and also for catalase test. Mohan *et al*. (1994) obtained similar results who reported that *P. multocida* gave consistent results for catalase and indole production test. All of the isolates were negative for methyl red test, urease activity and gelatin liquification test as observed by Chandrasekaran *et al.* (1981). Mohan *et al.* (1994) also obtained consistent results for urease test. Hydrogen sulfide was produced by all isolates (Aslam *et al.* 1988).

There are numerous classification and typing techniques of *P. multocida*. In this study serotyping techniques of Carter (1955) were followed. IHA technique was used for serotyping of the isolates, as the all capsule groups are type specific. However, there is a slight antigenic relationship between groups B and E (Carter, 1963). Results of IHA confirmed that only Carter type B (Robert type-I) is associated with the disease. Aslam *et al.* (1988) and Islam, (1975) reported that *P. multocida* Robert's type-1 is exclusively involved in all HS outbreaks in Pakistan and is being used for vaccine production. Our results are in line with their findings. Islam, (1975) also isolated the virulent strains of *P. multocida* from nasopharyngeal and tonsilar region of healthy buffaloes and cattle from nasal swabs.

ACKNOWLEDGEMENTS

This project was supported by Deanship of Scientific Research, College of Science, Research Center, King Saud University, Saudi Arabia.

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