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STATUS OF HAEMORRHAGIC SEPTICEMIA IN CATTLE AND BUFFALO IN UTTAR PRADESH, INDIA

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ABSTRACT

The investigation was undertaken to assess the status of haemorrhagic septicaemia (HS) in cattle and buffalo in Uttar Pradesh (India). Conventional methods were used for the diagnosis of HS and counter immunoelectrophoresis (CIE) method for epidemiological and capsular typing of *Pasteurella multocida,* the organism involved in HS. A total of 25 isolates were obtained and identified as *P. multocida* serotype B. The isolates were recognized into 12 biotypes on the basis of sugar fermentation tests thus indicating the involvement of multiple strains of *P. multocida* serotype B in the study area. Bipolar characteristic was exhibited in 25 samples (blood and impression smears of vital organs). The presence of antibodies against *P. multocida* B:2 in 10 (22.2%) serum samples by CIE showed the prevalence of *P. multocida* B:2 infection in the area. The results indicated that conventional methods (bipolar staining and CIE) are useful for rapid epidemiological investigations of HS.

Keywords: Biotypes, bipolar staining, Counter immuno-electrophoresis, haemorrhagic septicaemia, *Pasteurella multocida*

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute, fatal disease of cattle and buffaloes, caused by *Pasteurella multocida* type B:2, B:2,5 and B:5 in Asian countries and in African countries, type E:2. Various other serotypes, namely A:1, A:1,3, A:3, A:4, B:1, B:2,5, B:3,4, E:2,5, F:3 and F:3,4 have also been isolated from HS outbreaks (De Alwis, 1999; Kumar *et al.*, 2004, 2009; Shivachandra *et al.*, 2011). The disease is common to all wet tropical countries in Asia and still remains as one of the most serious animal diseases with 100% mortality in infected animals leading huge economic losses to livestock farmers (De Alwis, 1999; OIE, 2009). Singh *et al*. (2008) calculated the annual economic losses in India due to *P. multocida* infection alone to the tune of ₹ 225 millions. But, due to poor reporting and surveillance systems, losses due to HS are actually greater than that have been published. OIE World Organization for Animal Health (OIE) classified HS under list B diseases.

Pasteurella multocida is present as a commensal in respiratory tract of the animals which are clinically normal, and hence the primary relationship of the organism to the disease has frequently been in doubt. Normally these organisms infect the animal following exposure to debilitative condition and

predisposing factors (Rimler, 1984). Haemorrhagic septicaemia is present all the states of India and high risk area in India are the parts of Rajasthan, Gujarat, Karnataka, Andhra Pradesh, Bihar and Assam. In the present study, *P. multocida* originating from cattle and buffaloes were studied with respect to their ability to ferment different sugars for biotyping and their prevalence among cattle and buffaloes and capsular typing by CIE test in Uttar Pradesh, India.

MATERIALS AND METHODS

Sampling and isolation of *P. multocida:* A total of 400 samples from apparently healthy animals, clinical cases and dead animals supposed to be died of HS were collected. Out of them, 335, 55 and 10 samples were from healthy, clinical cases and dead animals, respectively, from different places like organized dairy farms, veterinary hospitals and villages of Uttar Pradesh, India. The samples were collected directly as blood from jugular vein at high rise of temperature in a sterile test tube with EDTA aseptically or from nasal mucosa by sterile swabs (live animals) and heart blood, lung pieces, liver and spleen aseptically from dead animals after post-mortem and carried to laboratory on ice. The samples were immediately inoculated into blood agar (HiMedia, India) and incubated aerobically at 37^{o}C for 24 h. The well-separated colonies were picked up onto blood agar slants as pure culture and subjected to standard morphological, biochemical and sugar fermentation tests as per Cowan and Steel (1993) to ascertain their identity as *P. multocida*. Besides, the morbid material and blood was used for the preparation of impression smear and stained with Leishman's stain to ascertain bipolar characteristic of the organism. Moreover, 43 blood samples were used for the separation of serum and two spleen tissue samples from dead animals were used as antigen.

Pathogenicity test: Twenty five rabbits were obtained from the Departmental laboratory rearing section. Each isolated *P. multocida* were grown for 18 h in an incubator at 37^{o}C in BHI broth. About 0.5 ml of each culture containing 2.4 x 10^{8} CFU ml^{-1} was inoculated into each rabbits by subcutaneous route and observed for 72 h to study mortality pattern. Reisolation of the organism was carried out on blood agar plate with heart blood collected from dead rabbits and impression smear from liver was prepared for observation following Leishman's staining method. Culture was also inoculated on Mc Conkey's agar (HiMedia, India) for checking its purity.

Preparation of hyper-immune sera: Hyper-immune serum was prepared as per OIE manual (2009). Briefly, *P. multocida* B:2 (P_{52} strain of *P. multocida* obtained from the Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, India) was grown in casein sucrose yeast extract (CSY) blood agar medium and harvested by washed in PBS and then treated with 0.3% formalin and its turbidity was adjusted to McFarland's tube No. 4. A total of 6 injections were given to each rabbit through marginal ear vein at 3 days interval with a dose of 0.2, 0.5, 0.75, 1.0, 1.5 and 2.0 ml. One week after last injection a booster dose of 0.5 ml of live culture (18 h) was given and animal was bled after 10 days. The serum was collected and stored at -20ºC until use.

Rapid slide agglutination test: The test was performed as per OIE manual (2009). A single colony was mixed with a drop of normal saline solution (NSS) on a slide and a drop of standard antiserum procured from FAO Regional Reference Laboratory (Asian region), Peradeniya, Srilanka was added and slide warmed gently. A course floccular agglutination appeared within 30 sec.

Counter immunoelectrophoresis: The identification of *P. multocida* capsular types B and E was done as per Carter and Chengappa (1981). Capsular antigen of each isolate and *P. multocida* P_{52} was prepared by lawn culture grown on CSY blood agar plates. After incubation for 24 h the growth was washed off with 5 ml of NSS. The suspension was heated at 56°C for 30 min and suspension sedimented by centrifugation. The clear supernatant fluid was used as the capsular substance. The

electrophoresis plates were prepared by pre-coating the glass plates (10 x 8 cm) with 15 ml volumes of coating medium (0.025 M barbitone buffer, 0.015% sodium azide, 0.5% bacto-agar and 0.5% agarose). Wells were cut 7 mm apart in a row and a parallel set of well was cut 6 mm away from the previous set.

Capsular antigen 20 µl per well was loaded at cathode side, while an equal volume per well of type specific antiserum was loaded at anode side. Negative rabbit serum and NSS were used as controls against capsular antigen and positive antiserum, respectively. The electrophoresis was carried out with barbitone buffer pH 8.8 (barbitone sodium, 9.75 g; sodium acetate, 3.9 g; 0.1 N HCl, 180 ml; distilled water 1 L) at 150 V for 30 min. The plates were examined for precipitation lines. Counter current electrophoresis was also used for sero-survillance of HS. Antigen prepared from *P. multocida* P_{52} was used against serum samples and hyperimmune serum against spleen tissues collected from clinical cases, dead and healthy animals.

RESULTS AND DISCUSSION

The diagnosis of infection is mainly depended on the isolation and identification of causative agents from clinical sample or dead animals. However, in case of HS, the preliminary diagnosis can be made upto genus level by staining the blood smears, exudates and infected animal tissues with Leishman's stain and observing the typical bipolar characteristic of organism. Out of 400 samples 25 (6.25%) exhibited bipolar characteristics in Leishman's stained blood smear or impression smear from pieces of lung, liver and spleen revealed presence of *P. multocida* (Fig. 1), from which 25 isolates recovered (Table 1). The fresh cultures, blood smears, exudates and infected animal tissues showed bipolar staining of organism with Leishman stain (OIE, 2009). Similarly, all 25 isolated *P. multocida* organisms showed bipolar staining in heart blood smear from rabbits died in pathogenicity test. These findings are similar to Sarma and Boro (1980). Bipolar staining of blood smear with Leishman's stain is a peculiar character of *P. multocida* organism used for on-spot diagnosis of disease and suggestive of *P. multocida* infection in animals (Sarma and Boro, 1980).

In the present investigation the isolation frequency of *P. multocida* was 4.5, 1.75 and 0.0% from the clinical cases, dead animals and healthy animals, respectively. The overall isolation percentage of *P. multocida* from the total cattle and buffaloes examined was 6.25% (25 isolates of 400 samples) (Table 1). The bacterial colonies on blood agar were small, round, smooth, non-haemolytic and dewdrop-like in appearance whereas no growth was observed on McConkey's agar. All the isolates were positive for indole and nitrate reduction tests. The organisms showed no reaction with citrate, MR and VP tests. The results of cultural and biochemical tests were in accordance with those of *P. multocida* (Dutta *et al*., 2001, Biswas *et al*., 2004, Kumar *et al*., 2009).

Table 1: Distribution of *P. multocida* isolated from various samples.

Samples	Total number of samples	Cattle	Buffalo	Bipolar staining	No. of Isolates	Percentage
Clinical cases blood	21	2	4	+	6	4.5
Clinical cases oedematous Fluid	34	4	8	+	12	
Lung	03	1	1	+	2	
Liver	03	1	1	+	2	1.75
Spleen	04	1	2	+	3	
Healthy animals (nasal swab/ blood)	335	0	0	0	0	0
Total					25	6.25

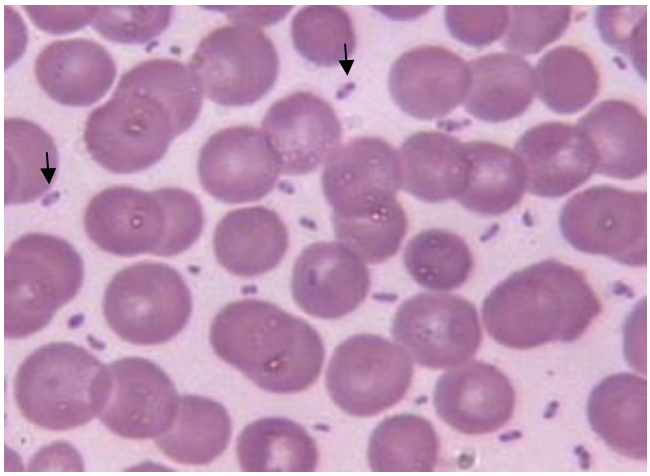

Fig. 1: Leishman's stained blood smear showing bipolar organisms (Arrow indicates bipolar organisms)

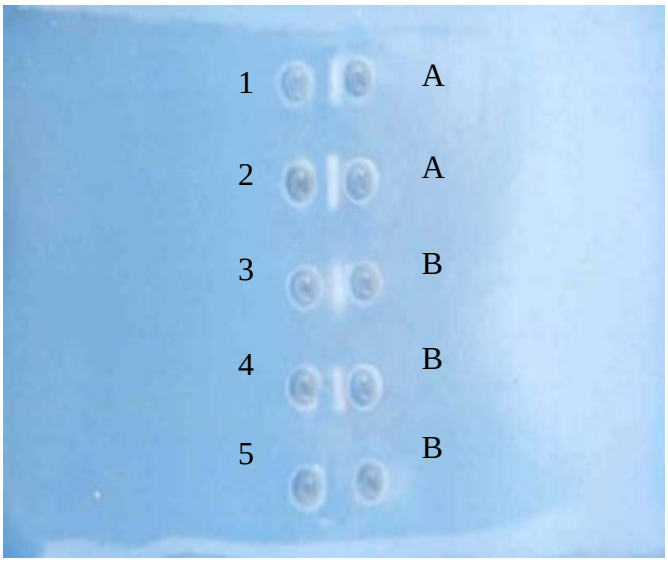

Fig. 2: Precipitin bands in counter current immuno-electrophoresis showing homologous antigen and antibody. Well No. 1: Antigen (clinical case blood sample from cattle), Well No. 2: Antigen (clinical case blood sample from buffalo), Well No. 3: Antigen (clinical case edematous fluid from buffalo), Well No. 4: Antigen (post mortem spleen from cattle), Well No. 5: Negative control-Normal saline solution. Well A: hyper immunesera from FAO regional reference laboratory (Asia Region) Sri Lanka. Well B: hyper immunesera prepared in laboratory against *P. multocida* P_{52}.

Biotyping is one of the trusted traditional methods for identifying bacterial species following primary isolation. In present study all 25 isolates were distributed into 12 biotypes by sugar fermentation reaction (Table 2). Majority of the isolates fermented glucose sugar, except 5 isolates, 19 isolates fermented mannitol and 18 isolates each fermented sucrose, sorbitol and maltose. Our observations are in conformity with Blackall *et al*. (1997), Biswas *et al*. (2004) and Kumar *et al*. (2009).

In pathogenicity testing all *P. multocida* isolates were found pathogenic to rabbit, causing 100% mortality within 24 to 48 h. The reisolated colonies showed characteristics of *P. multocida* colonies and impression smears revealed typical bipolarity of the organism. Death of all the rabbits used for pathogenicity test within 24 to 48 h indicated the virulence of organisms. These findings are in agreement with the observations of Seikh *et al*. (1994) and Butt *et al*. (2003). Each isolated organism was confirmed as *P. multocida* B:2 by demonstrating positive agglutination reaction with *P. multocida* B:2 standard antiserum.

Counter immunoelectrophoresis (CIE) has been preferred over indirect haemaglutination test (IHA) because it provides a simple and rapid procedure to detect microbial antigen including the capsular polysaccharide. A sharp precipitin line was formed against each 25 isolates (Fig. 2; Table 3) indicated that all the isolated organisms belonged to *P. multocida* B:2. The *P. multocida* type B specific capsular substance could be detected with CIE in the sera of rabbits, cattle and buffaloes with fatal type B infection suggesting that this procedure may be applicable for the laboratory diagnosis of HS. If the serum is not available tissue extract might be used as a source of antigen. Our observations are in line with those of Carter and Chengappa (1981) and Chengappa *et al.* (1986). In addition, out of 43 serum samples 10 (22.22%) were formed precipitin line with *P. multocida* B:2 antigen in CIE test and none of the spleen tissue samples produced precipitin line with standard antisera of *P. multocida* B:2 (Table 3).

Table 2: Identification of *Pasteurella multocida* biotypes

Biotype	Isolates (No.)	Glucose	Sucrose	Mannitol	Sorbitol	Lactose	Maltose	Agglutination
1	5	+	+	+	+	-	+	+
2	4	+	-	-	+	-	+	+
3	3	+	+	+	-	-	+	+
4	3	-	+	+	+	-	-	+
5	2	+	-	+	+	-	+	+
6	2	+	+	+	-	-	-	+
7	1	+	-	+	+	-	+	+
8	1	-	+	+	-	-	+	+
9	1	+	+	-	-	-	+	+
10	1	+	+	+	+	-	-	+
11	1	-	+	+	+	-	+	+
12	1	+	+	+	-	-	-	+
Total	25							

Table 3: Sero-surveillance of sera samples collected from healthy animals, clinical and HS cases

Animal species		No. of Samples	
		Serum (positive/ No. of samples)	Spleen (positive/ No. of samples)
Cattle	Healthy	1/12 (8.33)	0/1
	Clinical cases	3/4 (75)	0/0
	Dead	0/0 (0)	0/1
Buffalo	Healthy	2/19 (10.53)	0/0
	Clinical cases	3/6 (50)	0/0
	Dead	1/2 (50)	0/0
Total animals 45		10/43 (22.22)	0/2

The values in parenthesis indicate percent prevalence.

Conclusions: Based on the results of this study, it was concluded that CIE test and bipolar staining are rapid, simple and specific method for the identification of *P. multocida* isolates. Also, CIE is useful for diagnosis of HS in serum and tissue extract. All the isolated *P. multocida* were distributed into 12 biotypes thus indicating the involvement of multiple strains of *P. multocida* in the study area.

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