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RESTRICTION ENDONUCLEASE ANALYSIS OF *PASTEURELLA MULTOCIDA* FIELD ISOLATES BY Hha-I

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ABSTRACT

In this study the restriction endonuclease analysis (REA) technique was used for the comparison of field isolates and vaccinal strain of *Pasteurella multocida* using Hha-I enzyme. On the basis of DNA finger prints, extent of homology among the field isolates and the vaccinal strain was observed. The study has provided the clear evidence that eight isolates of *P. multocida* used were serologically different but genotypically all were same with the restriction enzyme used.

Key words: *Pasteurella multocida*, haemorrhagic septicemia, DNA fingerprinting.

INTRODUCTION

Haemorrhagic septicemia (HS) is an acute and often fatal disease principally occurring in cattle and buffaloes, but occasionally other domesticated and wild mammals can be affected (Carter and De-Alwis, 1989; De-Alwis, 1992). It is a primary pasteurellosis caused by *Pasteurella multocida* and manifested by an acute and highly fatal septicemia. Radical changes in weather, including the advent of monsoon, debility caused by seasonal level of low nutrition and pressure of work (Draught animals) are related to the explosive occurrences of the disease in certain parts of the world (De-Alwis, 1999). South Asia, including Pakistan, where such conditions often coincide is the area of highest incidence. The disease also occurs in Middle East and Africa where environmental circumstances and predisposing conditions are not as clearly defined as in Southeast Asia (De-Alwis, 1999). Specific strains of *P. multocida* namely; B:2, B:2,5 and E:2, E:2,5 have been detected from HS cases in Asia and Africa, respectively.

Serological identification of *P. multocida* is based on capsular (A, B, D, E and F) and somatic antigens (1-16) (Carter, 1955; Heddleston *et al*., 1972). Sero-groups B and E of *P. multocida* are distinguished by indirect haemagglutination and rapid slide agglutination tests and by their inability to be affected by certain muco-polysaccharides (Rimler and Rhoades, 1989; Rimler and Wilson, 1994). Somatic serotypes are distinguished by agar gel diffusion precipitation test (Rimler and Rhoades, 1989). Although serological and biochemical tests have proved to be useful for the detection and identification of organism but these techniques are limited, as they provide insufficient information for the epidemiological purposes where discrimination among different strains of the same serotype is important. There is also an inconsistency between the typing systems (Brogden and Packer, 1979). In addition, reports indicate that the significant proportion of isolates is un-typeable (Namioka and Bruner, 1963; Manning, 1982). In recent years, molecular techniques have been applied increasingly to discriminate among the isolates of *P. multocida*. Among these techniques, restriction endonuclease analysis (REA) is capable of recognizing differences in bacteria within the same serotype (Wilson *et al*., 1992).

In Pakistan, HS is considered as a disease of severe economic importance. Only in Punjab, the losses due to HS are 2.17 billion Pakistani rupees (Anonymous, 1996). Pakistan has a cattle population of about 24.2 million and a buffalo population of 26.3 million heads (Pakistan Economic Survey, 2004-05). The time trend suggests that the course of the disease is changing in Pakistan and high rates of morbidity and mortality have been recorded sometimes in vaccinated animals. This necessitates the characterization of organism. This study describes the conventional serotyping by REA of different field isolates of *P. multocida* associated with HS.

MATERIALS AND METHODS

Source of bacteria

Eight isolates of bacterium *P. multocida*, already isolated from overt cases of HS, were recovered from the stocks kept at -20°C in lyophilized conditions of Bacteriology Laboratory of Animal Health Institute, National Agriculture Research Centre, Islamabad, Pakistan (Table 1). Lyophilized *P. multocida* isolates were suspended in normal saline and revived in bulb/c mice by giving subcutaneous injection. The mice died after 24 hours and their hearts were collected asceptically (Buxton and Fraser, 1977).

Culturing of *P. multocida*

Mice heart blood for each isolate was inoculated on tryptone soya agar (Oxoid Ltd., Basingstoke, Hampshire, England) and was incubated at 37°C for 24 hours. After incubation, the colonies were subcultured

Table 1: Source of various isolates

Isolate No.	Locality
I	Veterinary clinic, Khushab
II	Veterinary Research Institute, Lahore
III	Veterinary clinic, Peshawar
IV	Veterinary clinic, Sheikhupura
V	Veterinary clinic, Islamababad
VI	Veterinary clinic, Gilgit
VII	Veterinary clinic, Qadirabad
VIII	Veterinary clinic, Karachi

for the purity. The bacteria from the purified colonies were confirmed morphologically and biochemically using "API 20® NE" kit (BioMérieux, Inc, Durham, USA), as described earlier (Wilson *et al*., 1993).

Sero-typing of the isolates

Rapid slide agglutination test was performed by using capsular type B antiserum for the capsular sero typing of the isolates. The capsular type B antiserum was obtained from National Veterinary Laboratories, Ames, Iowa, USA, (Hudson, 1959). For the somatic serotyping of the isolates, core antigen was extracted and the isolates were tested against the reference antigen by AGPT (Heddleston *et al*., 1972).

DNA fingerprinting

P. multocida isolates were grown in brain heart infusion broth at 37°C for 24 hours. An amount of 1 ml culture was centrifuged in eppendorf tube at 16,000 g for 2 minutes and the supernatant was poured off. The process was repeated four times to pellet down more cells in the same eppendorf tube.

These cells were suspended in 1 ml of Tris-EDTA (TE) buffer and re-centrifuged at 16,000 g for 2 minutes. The supernatant was poured off except for 50-75 ul of it. TE buffer (350 ul) was added to the cells and vortexed until the cells were completely suspended. A 150 ul lysozyme (Sigma, St-Louis, USA) was added and mixed. The tubes were placed on ice for 30 minute and white clumping appeared in the tube. Sodium dodecyl sulfate (10%, 40 ul) was added in the tube and mixed until suspension was cleared. Then 8 ul of RNase (Sigma, St-Louis, USA) was added and mixed till the precipitate in the tube cleared away. Then 60 ul of proteinase K (Sigma, St-Louis, USA) was added and mixed.

The tubes were incubated at 37°C for one hour in water bath. An amount of 0.8 ml of TE saturated phenol was added and mixed till the mixture turned white. It was then centrifuged at 13,000 rpm for 2 minutes. Aqueous phase (600 ul) was taken out and transferred into a clean eppendorf tube. Then 0.8 ml of chloroform/isoamyl alcohol was added to the new tube, the tube contents were mixed and again centrifuged at 13,000 rpm for 2 minutes. A 375 ul of aqueous layer was removed and taken in a tube having 75 ul of sodium acetate and mixed by gentle pipetting. Then 1 ml of Ice-cold ethanol (95%) was added in the tube. After inverting the tube 5 or 6 times, the mixture was left at -20°C overnight and centrifuged at 12,000 rpm for 10 minutes. The supernatant was decanted, pellet obtained was air dried, re-dissolved in 100 ul of double distilled water and stored at -20°C (Wilson *et al*., 1993).

Restriction Endonuclease Analysis

The purified DNA samples from eight different isolates were subjected to REA. These DNA samples were digested with Hha-I Endonuclease (Invetrogen™, USA) and subjected to electrophoresis in 0.8% agarose gel at 100 V and 30 mA. Buffer used in electrophoresis was 0.5% tris boric acid ethylenediamine tetraacetic acid. A Hind III digest of lambda phage (Sigma, St-Louis, USA) was used as standard for the comparison of fingerprint profiles. The gel was stained with ethidium bromide for 15 minutes with constant shaking. The gel was rinsed once with distilled water and was photographed in the presence of UV illuminance (Wilson *et al*., 1992).

RESULTS AND DISCUSSION

Morphologically, all the isolates were Gram negative coccobacilli. Bio-chemically, all the isolates reduced nitrate, decomposed tryptophan and produced oxidase. Negative results were shown by the isolates for the glucose acidification, hydrolosis of gelatin and asculin. The isolates were also negative for the presence of β-glactosidase in AUX medium (Table 2).

The results obtained from all the tests were transformed into a numerical data by filling up the result sheet, which was 3000 004. This value was checked in the analytical profile index. The value corresponded to *P. multocida* with a 93 percent identification that was considered as a good identification. API 20®NE identification system was found to be an effective and rapid method of biochemical characterization of the isolates and has been used in the previous studies with *P. multocida*. Its disadvantage is lack of consistency among the percentage of identification e.g., 100 percent was found for *P. multocida* sub species septica (Boerlin *et al*., 2000), 96.9 percent was observed for *P. pneumotropica* (Frebourg *et al*., 2002) using API 20®NE system. Difference of serotype is basis for this variation. Metabolic activity of isolates of the similar serogroups may be different, resulting in variation in identification. In addition, API 20®NE and API 20®E also give different identification percentage even for the same organism.

Pasteurella multocida isolates were typed for their capsular antigen by rapid slide agglutination test. HS in cattle, buffalo and other animals like deer and elk have been associated with *P. multocida* Carter's serogroup B (Carter, 1955; Aalbaek *et al*., 1999). All the isolates showed agglutination against serotype B. For the somatic typing of the isolates, core antigen was extracted and tested against the reference antigen by AGPT, using method described by Heddleston *et al*. (1972). Five of the eight isolates designated as II, IV, VI, VII and VIII isolated from VRI Lahore,

Table 2: Biochemical characteristic of *Pasteurella multocida* field isolates

Test No.	Biochemical tests	Reactions	Isolates							
			I	II	III	IV	V	VI	VII	VIII
1.	NO_3	Reduction of nitrates	+	+	+	+	+	+	+	+
2.	TRP	Indole reduction	+	+	+	+	+	+	+	+
3.	GLU	Acidification	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4.	ADH	Arginin dihydrolase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5.	URE	Urease	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6.	ESC	Esculin hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7.	GEL	Gelatin hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8.	PAC	Phenyl acetate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Assimilation of sugars										
9.	PNPG	β-glactosidase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10.	GLU	Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
11.	ARA	Arabinose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
12.	MNA	Mannose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
13.	MAN	Mannitol	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
14.	NAG	N-acetyl glucosamine	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
15.	MAL	Maltose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
16.	GNT	Gluconate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
17.	CAP	Caprate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
18.	ADI	Adipate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
19.	MLT	Malate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
20.	CIT	Citrate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
21.	OX	Cytochrome oxidase	+	+	+	+	+	+	+	+

Sheikupura, Gilgit, Qadirabad and Karachi, respectively, gave positive results with reference strain 2 and 5, while the remaining isolates I, III and V from Khushab, Peshawar and Islamabad, respectively, did not react with the antisera 5 (Table 3).

Pasteurella multocida is identified from its somatic and capsular antigens. Isolates identified as 2,5 could belong to any of the 5 capsular groups. Detection of somatic antigens was done by somatic antisera prepared and evaluated with reference somatic strains. Isolates expressing multiple somatic antigens can mislead the diagnosticians and research scientists.

Table 3: Sertypes of *P. multocida* field isolates

Isolate No.	Locality	Capsular type (Rapid slide agglutination test)	Somatic serotype (AGPT)
I	Veterinary clinic, Khushab	B	2
II	Veterinary Research Institute, Lahore	B	2,5
III	Veterinary clinic, Peshawar	B	2
IV	Veterinary clinic, Sheikhupura	B	2,5
V	Veterinary clinic, Islamabad	B	2
VI	Veterinary clinic, Gilgit	B	2,5
VII	Veterinary clinic, Qadirabad	B	2,5
VIII	Veterinary clinic, Karachi	B	2,5

Serological identification depends on factors like production of anti sera, problems of cross reactivity and variation in immunological responses between animals. These factors pose problem for standardization. Still serotyping procedures are important for diagnostic point of view. Conventionally, both somatic and capsular serotypings are done before protein profiling. During studies like expression of iron regulated outer membrane proteins, analysis of protein patterns of *P. multocida* which are used to differentiate the strains require specification of the serotype first (Ruffalo *et al*., 1997; Kedrak and Opacka, 2002).

All the isolates were found to be Gram-negative coccobacilli after the Gram's staining and *P. multocida* was confirmed by API 20®NE. Rapid slide agglutination test was performed for typing the isolates for their capsular antigen. All the isolates showed agglutination with the serum raised against serotype B and after the analysis all the isolates were designated as capsular type B. The results of the present study have been found in correlation with the previous studies where HS in cattle, buffaloes and in some other mammals like deer and elk have been associated with *P. multocida* Carter's serotype B (Carter, 1955; Aalbaek *et al*., 1999).

Serological identification depends on factors like production of antisera, problem of cross reactivity and variation in immunological responses between animals.

These factors, therefore, cause a problem of standardization. However, the serotyping procedures are important from diagnostic point of view. There are various reports where both capsular and somatic serotypings have already been performed prior to molecular characterization. Molecular studies including DNA fingerprinting, ribotyping or even PCR, which are used to differentiate the strains, require specification of the serotype first (Harel *et al*., 1990; Wilson *et al*., 1992, 1993; Townsend *et al*., 1998; Aalbaek *et al*., 1999; Amonsin *et al*., 2002). Therefore, after serotyping, this study was extended to DNA fingerprinting.

Comparison of DNA fingerprints profiles

Each of the somatic serotypes of *P. multocida* produced fingerprint profile upon digestion with Hha I. A previous study showed that REA with Hha-I endonuclease could discriminate among isolates of the serogroup B of *P. multocida* that can cause HS (Wilson *et al.,* 1992). The current study, with different isolates of *P. multocida* obtained from different localities of Pakistan, showed that REA with Hha-I endonuclease could result in better discrimination among these bacteria.

All serotype B:2 fingerprint profiles were characterized by two tailing bands at ~8.4-6 kb after digestion with Hha-I endonuclease and electrophoresis. A profile characterized by these particular bands has not been seen in any other *P. multocida* serotype isolated with the same endonuclease and thus, like the ability to produce hyaluronidase, may constitute another property that can be used as a diagnostic test to recognize members of the B:2 serotype. As determined by percentage similarity of the fingerprint profiles, the B:2 serotype constituted a more homogeneous cluster than that seen with the other HS serotypes tested (B:3,B:4 and B:3,4) (Rimler, 2000).

Hha-I yielded fingerprint profiles that were most easily and best distinguished, therefore, all primary digestion studies were done with this endonuclease (Wilson *et al*., 1992). In this study, DNA fingerprinting was examined as a possible method for further differentiation of strains. DNA fingerprinting has been used frequently for characterization of bacterial DNA. The efficiency and timeliness of DNA extraction and precipitation are commonly encountered problems. REA is a sensitive and relatively uncomplicated method of DNA fingerprinting that directly detects differences between strains of *P. multocida* which appear similar by traditional serotyping methods. Detection of differences with a small number of strains can be accomplished without much difficulty by visual comparison of profiles within a gel (Rimler, 2000).

Data obtained in this study have provided the clear evidence that eight isolates of *P. multocida* used were serologically different but genotypically these all were same with the restriction enzyme used. These results have been confirmed by the previous studies where both B:2 and B:2,5 were associated with HS and did not show any difference in their DNA fingerprint profiles (Wilson *et al*., 1992, 1993).

These results, therefore, suggest that the study should be extended by using more restriction enzymes to get unique DNA fingerprint profile to make sure the genotyping of the isolates and carrying similar studies by including more *P. multocida* isolates from areas that are severely affected by HS. Further efforts in getting unique genotypic profiles for HS causing isolates will certainly help in establishing the basis for selection for a better vaccinal *P. multocida* strain, thus overcoming this fatal disease in promising way.

Restriction endonuclease analysis, in combination with the conventional serotyping, could be a powerful tool for strain differentiation. It can be of great importance in epidemiological research that may lead to overcome HS.

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