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Molecular typing of haemorrhagic septicaemia-associated *Pasteurella multocida* isolates from Pakistan and Thailand using multilocus sequence typing and pulsed-field gel electrophoresis

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ABSTRACT

A comparative genetic study of 23 field isolates and vaccine strains of *Pasteurella multocida* associated with haemorrhagic septicaemia cases from Pakistan and Thailand was done using pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The MLST sequence type (ST) for all 20 of the 23 isolates tested was 122. The PFGE results showed one band difference between the Pakistani and the Thai isolates. Sequence type 122 is the dominant associated profile with haemorrhagic septicaemia (HS) cases in South Asia. The study supports the concept of using PFGE for short-term epidemiology and MLST for long-term epidemiology.

1. Introduction

Pasteurella multocida is a Gram-negative nonmotile and nonspore-forming rod or coccobacillus (Rimler and Rhoades, 1989). It is serologically classified into five Carter capsular groups, namely A, B, D, E and F (Carter, 1963; Rimler and Rhoades, 1987) and sixteen Heddleston somatic types (Heddleston et al., 1972). Different serotypes of *P. multocida* are associated with a variety of diseases in animals, such as fowl cholera (mainly serotypes A:1, A:3 and A:4) and atrophic rhinitis in pigs (toxigenic strains of serogroup D) (De Alwis, 1999, pp. 11–27).

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease in cattle and buffaloes associated with strains of serotypes B:2 (Asian serotype) and E:2 (African serotype) of the bacterium *P. multocida* (De Alwis, 1999, pp. 11–27), however HS-associated B:2 serotypes may occur in Africa, and E:2 serotypes may be found in Asia (Dziva et al., 2008).

Asia and Africa are currently the global geographic regions in which HS occurs with the highest prevalence and has the greatest economic importance (Benkirane and De Alwis, 2002). This is attributed to pronounced changes in weather between seasons, including the monsoon, debility caused by seasonal scarcity of fodder and pressure of work (draught animals) (Benkirane and De Alwis, 2002).

Haemorrhagic septicaemia has been reported to be the most important bacterial disease of cattle and buffaloes in Pakistan, and is considered a disease of great economic importance (Benkirane and De Alwis, 2002). In 1978, Pakistan reported that 34.4% of all deaths in buffaloes and cattle were due to HS and the estimated annual economic losses were 1.89 billion rupees (Benkirane and De Alwis, 2002). In Thailand, HS ranks highly on the list of economically significant diseases of cattle and buffaloes (Patten et al., 1993).

Molecular typing of different bacterial isolates is an essential molecular epidemiological tool. Different DNA typing methods such as restriction endonuclease analysis (REA), ribotyping, PCR methods, such as Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR, Repetitive Extragenic Palindromic (REP) PCR, Random Amplification of Polymorphic DNA (RAPD) PCR and pulsed-field gel electrophoresis (PFGE) have been used for genotyping of *P. multocida* (Blackall and Miflin, 2000). Multi locus enzyme

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electrophoresis (MLEE) (Blackall et al., 1998) and multi locus sequence typing (MLST) (Subaaharan et al., 2010) have also been used for molecular epidemiology. MLST is a portable and reproducible sequence based approach to molecular genotyping which facilitates the comparison of results from different laboratories (Maiden et al., 1998). PFGE has shown a great discriminatory power to investigate individual outbreaks in one geographic area so it is considered a gold standard for short term epidemiology (Maiden et al., 1998). On the other hand, MLST has been claimed to be the gold standard for studying long term (global) epidemiology (Maiden et al., 1998).

A MLST scheme has been developed for *P. multocida* called the Rural Industries Research and Development Corporation (RIRDC) *P. multocida* MLST scheme (Subaaharan et al., 2010). This database was developed initially for avian isolates and all the primer information, PCR conditions, allele sequences, sequence types and isolate information are available at the RIRDC *P. multocida* MLST website (http://pubmlst.org/pmultocida_rirdc/) sited at the department of Zoology, University of Oxford (Jolley et al., 2004). Presently, this database contains DNA sequences of *P. multocida* isolates from many hosts and from different pasteurelloses. Another scheme exists, called the *P. multocida* multi-host MLST scheme but it is not accepting submissions nowadays. (http://pubmlst.org/pmultocida_multihost/).

In this study we investigated the relatedness of HS-associated isolates of *P. multocida* within and between two HS-endemic countries in South Asia, namely Thailand and Pakistan. We also compared the discriminatory power of MLST and PFGE for the purpose of detecting genetic differences between these various isolates.

2. Materials and methods

A total of 20 field isolates and 3 vaccine strains previously identified as *P. multocida* were used in this study ($n = 23$). Twelve Pakistani field isolates and the Peshawar vaccine strain were collected from the National Veterinary Laboratory (NVL), Islamabad. The Lahore vaccine strain (B:2 serotype) was collected from the Veterinary Research Institute in Lahore. An additional 9 isolates (8 field strains and 1 vaccine strain) were received from the Department of Livestock Development (DLD), Ministry of Agriculture and Cooperatives, Thailand (Table 1).

2.1. Biochemical characterisation

Isolates 1–20 were characterised biochemically using the Microbact Gnb 24E tray (Oxoid, UK, Cat# MB1131A), according to the manufacturer's instructions. Briefly, each isolate was grown on brain heart infusion (BHI) agar (Merck Chemicals) for 24 h at 37 °C and then 2–3 pure colonies were inoculated in 5 mL sterile saline (0.85%) containing 5 drops of sterile foetal bovine serum. Motility was checked for all isolates by stab inoculation into a Tryptone Soya slant (semisolid medium) (Oxoid, UK, Cat# CM0131). The results were interpreted after incubation for 48 h at 37 °C.

Each isolate was also tested for oxidase production using oxidase test strips (Oxoid, UK, Cat#MB0266), and catalase production using hydrogen peroxide.

An octal number was assigned to each isolate and then interpreted using the Microbact computer aided identification package (MB1244A). The results of oxidase test, motility and nitrate reduction were added to this octal number to produce a nine digit code which was entered into the software program.

2.2. DNA extraction

Genomic DNA of *P. multocida* was extracted using the Purelink genomic DNA Kit (Invitrogen Cat# K182001). The protocol for Gram negative bacteria as described in the kit was followed for extractions.

Table 1
Isolates used in the study.

Isolate number	Isolate ID	District	Province	Country	Location coordinates	Species	Year	RIRDC MLST accession number	PFGE results (Fig. 1)
1	Sahiwal	Sahiwal	Punjab	Pakistan	30.66°N, 73.11°E	Buffalo	2008	545	ND
2	Bhakkar	Bhakkar	Punjab	Pakistan	31.63°N, 71.07°E	Cattle	2008	548	ND
3	Attock	Attock	Punjab	Pakistan	33.91°N, 72.31°E	Cattle	2010	546	Lane 10
4	Karachi 1	Karachi	Sindh	Pakistan	24.86°N, 67.01°E	Cattle	2007	551	ND
5	Karachi 2	Karachi	Sindh	Pakistan	24.86°N, 67.01°E	Buffalo	2011	552	ND
6	Karachi 3	Karachi	Sindh	Pakistan	24.86°N, 67.01°E	Buffalo	2011	553	Lane 11
7	Peshawar	Peshawar	Khyber Pakhtunkhwa	Pakistan	34.02°N, 71.58°E	Buffalo	2011	556	Lane 12
8	Islamabad 1	Islamabad	Islamabad capital territory	Pakisatn	33.72°N, 73.07°E	Wild buffalo	2011	554	ND
9	Islamabad 2	Islamabad	Islamabad capital territory	Pakistan	33.72°N, 73.07°E	Buffalo	2012	555	ND
10	Jhelum	Jhelum	Punjab	Pakistan	32.93°N, 73.73°E	Buffalo	2009	547	ND
11	Taxila 1	Rawalpindi	Punjab	Pakistan	33.75°N, 72.79°E	Buffalo	2012	549	ND
12	Taxila 2	Rawalpindi	Punjab	Pakistan	33.75°N, 72.79°E	Buffalo	2012	550	ND
13	Lahore vaccine strain	Lahore	Punjab	Pakistan	31.55°N, 74.34°E	Buffalo	2011	558	Lane 14
14	Peshawar vaccine strain	Peshawar	Khyber Pakhtunkhwa	Pakistan	34.02°N, 71.58°E		2011	557	Lane 13
15	Thailand A	Thung song	Nakhon Si Thammarat	Thailand	8.16°N, 99.68°E	Buffalo	2006	539	Lane 1
16	Thailand B	Mueang Phitsanulok	Phitsanulok	Thailand	16.82°N, 100.26°E	Buffalo	2011	540	Lane 2
17	Thailand C	Rong Kham	Kalasin	Thailand	16.27°N, 103.74°E	Buffalo	2006	541	Lane 3
18	Thailand D	Phanat Nikhom	Chonburi	Thailand	13.45°N, 101.18°E	Buffalo	2009	542	Lane 4
19	Thailand E	Chiang Dao	Chiang Mai	Thailand	19.37°N, 98.96°E	Buffalo	2009	543	Lane 5
20	Thailand F	Mueang Lamphun	Lamphun	Thailand	18.58°N, 99.02°E	Buffalo	2011	544	Lane 6
21	Thailand G	Warin Chamrap	Ubon Ratchathani	Thailand	15.20°N, 104.87°E	Buffalo	2008	ND	Lane 7
22	Thailand H	Mueang Khon Kaen	Khon Kaen	Thailand	16.44°N, 102.84°E	Buffalo	2010	ND	Lane 8
23	Thai HS vaccine strain	NA	NA	Thailand	NA	Buffalo	NA	ND	Lane 9

ND = not done.
NA = not applicable.