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Molecular Characterization Indian Isolates of *Pasteurella multocida* Isolated from Buffalo and Cattle in India

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A B S T R A C T

The applicability of repetitive palindromic sequences based differentiation of *Pasteurella multocida* serogroup B isolates involved in outbreak of haemorrhagic septesemia (HS) in Tarai region of India was evaluated. Seven isolates of *Pasteurella multocida* B: 2 were isolated from buffaloes and cattle. These were subjected to phenotypic and genotypic characterization. PCR assays were used for the characterization of these isolates. Repetitive extragenic palindromic REP- PCR and Enterobacterial repetitive intergenic consensus ERIC- PCR differentiated all the seven isolates into six profiles. All the isolates were found genetically distinct from standard *Pasteurella multocida* strain P52 (Vaccine strain of India).Two isolates shared same profile while all other isolates shown different profiles. This study provides a clear evidence of presence of more than one isolate in single outbreak of HS and also provide indication of high genetic variation among field isolates of *Pasteurella* and may be the reason of vaccine failure and outbreaks.

Introduction

Haemorrhagic Septicaemia (HS) is an important disease cattle and buffalo in India. It is by *Pasteurella multocida* types B: 2, B: 2, 5 and B: 5 and has high mortality rate. Conventional methods of disease diagnosis are time consuming as it requires isolation identification and differentiation through bacteriological techniques. With the advancement of molecular biology Polymerase chain reaction (PCR)-based techniques has been extensively used for detection and differentiation of pathogens (Shivshankar *et al.*, 2001; Saxena *et al.*, 2006; Shivchandra *et al*., 2006; Saxena *et al*., 2004, Nagagapa *et al.*, 2007). HSB-PCR (Haemorrhagic Septicaemia serotype B specific PCR) is rapid, specific and highly sensitive, are being employed efficiently for early detection (Towensend *et al.*, 1998) and differentiation of various isolates of *P. multocida* B:2 from single and different outbreaks (Biswas *et al.*, 2004).

In the present study HS suspected animals were screened using HSB-PCR. Seven isolates were isolated from affected animals (Cattle and Buffalo). These were characterized by using conventional (biochemical, pathogenicity testing, antibiotic analysis) and molecular markers. Two DNA markers were used these were Enterobacterial repetitive intergenic consensus sequence (ERIC) and Repetitive extragenic palindromic sequence (REP) these are repetitive sequences present in family Enterobacteriacae. Out ward primers for these sequences were developed by Verosalovic *et al*. 1991 and field isolates of *Pasteurella multocida* were differentiated on basis of copy number and inter sequential distances (Loubinoux *et al*., 1998; Saxena *et al*., 2006).

In present study investigations was carried out during an outbreak of haemorrhagic septesemia to study genetic variation among field isolates using conventional and molecular markers with respect to design a strategic control measures.

Materials and Methods

Screening of samples by HSB-PCR

Preparation of culture lysate

Blood samples (5 ml each) and nasal discharges were collected from affected animals and healthy. Samples were inoculated in 2 ml of BHI broth for 18 hrs at 37^0C. 1.5 ml of 18hrs growth was subjected for centrifugation. Pellet was resuspended in 100µl sterilized triple distilled water. Then tubes were boiled at 100^0C for 10 minutes and chilled immediately in ice. The tubs were centrifuged at 5000 rpm for 10 minutes to remove cell debris. 5µl of the supernatant was used as template for 25 µl of PCR reaction.

HSB-PCR

PCR was conducted by using KTSP 61 and KTT 72 primers (Towensend *et al.*1998) for the detection of *Pasteurella multocida* serotype B. 25 µl reaction mixture containing 5 µl of lysate, 1X PCR buffer, 1.5 mM MgCl2, 200 µM of each of dNTPs, 20pmol of each primer and 1 U Taq DNA polymerase was prepared. All PCR reagents were procured from MBI-Fermentas .PCR was performed with an initial denaturation step (94^0C for 5 min) followed by 30 cycles of denaturation (94^0C for 1 min), annealing (55^0C for 1 min) and extension (72^0C for 1 min, with a final) extension at 72^0C for 10 min.

Amplified product were separated by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 5V/cm for 2h and stained with ethidium bromide (0.5 µg / ml). DNA fragments were observed by UV transilluminator and images were stored using gel documentation system (AlphaImager 2200 Documentation and Analysis System, Alpha Innotech Corporation, USA).

Isolation of *P. multocida* serotype B from positive samples

Positive lysates were inoculated in mice by intraperitoneal route. Heart blood was collected from died mice and streaked on plate of blood agar. Isolated single colony was used for methylene blue staining and HSB-PCR.

Biochemical test

Each isolate was tested for oxidase, indole and catalase tests. Growth of colony was observed on McConkey's agar and on blood agar. All the isolates were tested for fermentation of sugars glucose, fructose,

maltose, mannitol, sucrose and lactose. The isolates were tested for utilization of citrate, and gelatin.

Antimicrobial sensitivity test

Isolates were tested for their sensitivity against antimicrobial agents. The antibiograms was determined using a total of eleven different antibiotic discs (amoxycillin, 30 µg, pefloxacin, 5 µg, cephalexin, 30 µg, enrofloxacin, 10 µg, ciprofloxacin, 30 µg, streptomycin, 10 µg, gentamycin, 30 µg; erythromycin, 15 µg; oxytetracyclin,30 µg, chloramphenicol, 30 µg, sulphamithazole, 300 µg) on BHI agar according to disc diffusion method described by Carter and Subronto (1973).

Differentiation of isolates by ERIC-PCR and REP-PCR

Genomic DNA of all the isolates was extracted as per the C-Tab method described by Wilson (1987). REP-PCR was performed as per protocol described by Saxena *et al.*, (2006) with some variations. Briefly, 25µl reaction mixture containing 50ng of genomic DNA and 20pmol of each primer in 1X PCR buffer, 1.5 mM $MgCl_2$, 200µM of each of the four dNTPs and 0.5U Taq polymerase.PCR was performed with an initial denaturation step (94^0C for 5 min.)followed by 30 cycles of denaturation (94^0C for 1 min.), annealing (45^0C for 2 min.) and extension(72^0C for 6 min.), with a final extension at 72^0C for 10 min. Amplicons were loaded on 1.5% agarose and subjected for electrophoresis at 5V/cm for 2hrs. 100bp ladder was used as size marker. Fingerprints were stored in tagged-image filed (TIF) format with Bio ID software (Vilber Lourmat, Paris, France). Two fingerprints were considered identical if the same number of bands at the same positions were observed.

ERIC-PCR was also conducted as per described by Biswas *et al.*, (2004) with some variations. A reaction mixture of 25µl containing 50ng of genomic DNA and 20pmol of each primer in 1X PCR buffer, 1.5 mM MgCl2, 200µmol/l of each of the 4 dNTPs and 0.5U Taq polymerase.PCR was performed with an initial denaturation step (94^0C for 5 min.)followed by 30 cycles of denaturation (94^0C for 1 min.), annealing (45^0C for 2 min.) and extension(72^0C for 6 min.), with a final extension at 72^0C for 10 min. Amplicons were loaded on 1.5% agarose and subjected for electrophoresis at 5V/cm for 2hrs.

Results and Discussion

Isolation and phenotypic characterization

A total of seven strains were confirmed by HSB-PCR as *P. multocida*, serotype B these were designated P1, P2, P3, P4, P5, P6 and P7. All the seven isolates gave amplified products of ~620bp determining the products specific for HS causing serogroup B isolates (Figure 1).

The isolated bacterial colonies on blood agar plates were small, glistening, mucoid and dewdrop- like and appeared to be Gram-negative coccobacilli when stained with Gram's stain. Agglutination was produced on serological testing with antiserum raised against whole cell antigen of *P. multocida* (B:2).

All of the seven isolates were oxidase and catalase positive and urease negative. No growth was observed on McConkey's agar and were found to be non haemolytic on blood agar. All the isolates fermented glucose, fructose, maltose, mannitol and sucrose, but did not fermented lactose. The isolates were positive for indole test and no reaction was seen with citrate, and gelatin.

Antimicrobial sensitivity test

The results indicated that all the isolates exhibited drug resistance against amoxicillin and were sensitive for enrofloxacin followed by pefloxacin, ciprofloxacin, gentamycin, erythromycin, oxytertracyclin, choramphenicol, sulphamethazole, streptomycin, cephalexin.

In ERIC-PCR, amplified products were observed in all the isolates (Figure 2). However, out of the seven, five profiles were obtained and the products size varied from 128 to 2012. In ERIC type I isolates (P1 and P5) were present. In ERIC type II isolates (P4 and P6) were present. In ERIC type III, IV and V isolates P2, P3 and P7 were present respectively. Band of 488 bp were observed in all seven isolates.

REP-PCR analysis of all the seven isolates revealed five different profiles (Figure 3). In REP type I isolates (P2 and P3) were present. In REP type II isolates (P4 and P6) were present. In REP type III, IV and V isolates P1, P5 and P7 were present respectively.

Two bands of sizes 1080 and 784 bp were amplified in all the isolates. These may be *Pasteurella*-specific loci and can be exploited for the identification of *P. multocida.*

In both ERIC and REP-PCR P52 vaccine strain revealed entirely different profile. ERIC and REP profile data was analyzed using NTSYSpc v2.0 and dendogram was constructed. On combined typing out of seven, six different profiles were observed. P4 and P6 were showing common profile. (Fig. 4) .

In north India the disease assume to be epizootic during rainy season (June-September) (Khera, 1979) The attempts to isolates a causative agent from various animals resulted in isolation of a few *P. multocida* strain from cattle and buffaloes. Though more than hundred samples were collected but few isolates were isolated. The failure to isolate the organism from many cases could be due to administration of antimicrobial drug prior to sample collection. The isolates could not be differentiated on the basis of conventional methods involving culture, pathogenicity test and biochemical characterization. Earlier similar types of finding were reported from India (Biswas *et al.,* 2004).

Isolates revealed resistance for amoxicillin which is very commonly used in India. Though isolates were found to be sensitive for many other drugs (enrofloxacin) because these are costly and not so routinely used in field cases (Yoshimura *et al.,* 2001).

Table.1 Primers sequences used in the PCR technique for identification and characterization

S. No	Type of PCR		Sequences of primers used	References
1	HSB-PCR	KTT72 KTSP61	5'AGG CTC GTT TGG ATT ATG AAG-3' 5'ATC CGC TAA CAG ACT CTC-3'	Townsend *et al.* (1998)
2	REP PCR	REP1R 1Dt- REP2 1Dt-	5' NNN NCG NCG NCA TCN GGC-3' 5' NCG NCT TAT CNG GCC TAC-3'	Versalovic *et al.*(1991)
3	ERICPCR	ERIC 1R- ERIC2-	5' ATG TAA GCT CCT GGG GAT CAC-3' 5' AAG TAA GTG ACT GGG GTG AGC G-3'	Versalovic *et al.*(1991)

Table.2 ERIC PCR profile of field isolates of *Pasteurella multocida*

P1	P2	P3	P4	P5	P6	P7	P52
							2012
			1860		1860		1860
931		931		931		931	931
821		821	821	821	821		
		680					680
488	488	488	488	488	488	488	488
340		340	340	340	340	340	340
250		250		250		250	250
			170		170		
						128	128

Table.3 REP-PCR profile of field isolates of *Pasteurella multocida*

P1	P2	P3	P4	P5	P6	P7	P52
							3649
				2456			
2142	2142	2142					
1580	1580	1580					
						1147	
1080	1080	1080	1080	1080	1080	1080	1080
			899		899		
784	784	784	784	784	784	784	784
	446	446		446			446
	300	300				300	300

Table.4 Combined REP-ERIC profiles of isolates of *P. multocida*

Combined profiles

Strain no.	a	b	c	d	e	f	g	H	i	J	k	L	m	n	o	P	q	r	s	T
P1	0	0	1	0	0	1	0	1	1	0	1	1	0	1	0	1	0	1	0	0
P2	0	0	1	0	0	1	0	1	0	0	0	1	0	1	1	0	1	0	0	0
P3	0	0	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0
P4	0	0	0	0	1	0	0	1	0	1	1	1	0	1	0	1	0	0	1	0
P5	0	1	0	0	0	0	0	1	1	0	1	1	0	1	1	1	0	1	0	0
P6	0	0	0	0	1	0	0	1	0	1	1	1	0	1	0	1	0	0	1	0
P7	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0	1	1	1	0	1
P52	1	0	0	1	1	0	0	1	1	0	0	1	0	1	1	1	1	1	0	1

a, 3649 bp; b, 2456 bp; c, 2142 bp; d, 2012 bp; e, 1860 bp; f, 1580 bp; g, 1147 bp; h, 1080 bp; i, 931 bp; j, 899bp; k, 821 bp; l, 784 bp; m, 680 bp; n, 488 bp; o,446 bp; p, 340 bp; q, 300 bp; r, 250 bp; s, 170 bp; t, 128 bp; 0,band present; 1, band absent.

Fig.1

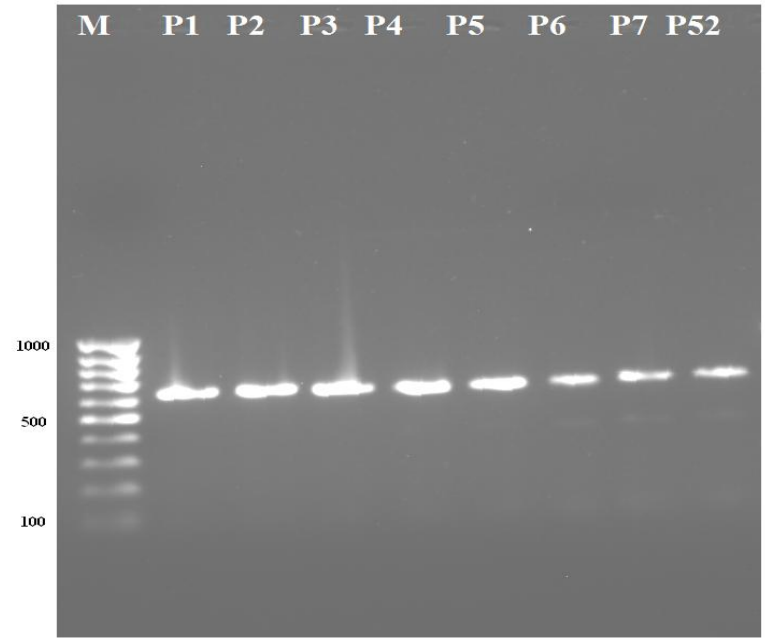


Figure1 . HSB PCR profiles. Lanes: M: Marker, 100 bp ladder; P1: isolate P1; P2: isolate P2; P3: isolate P3; P4: isolate P4; P5: isolate P5; P6:isolate P6; P7: isolate P7; P52: Vaccine strain.

Fig.2

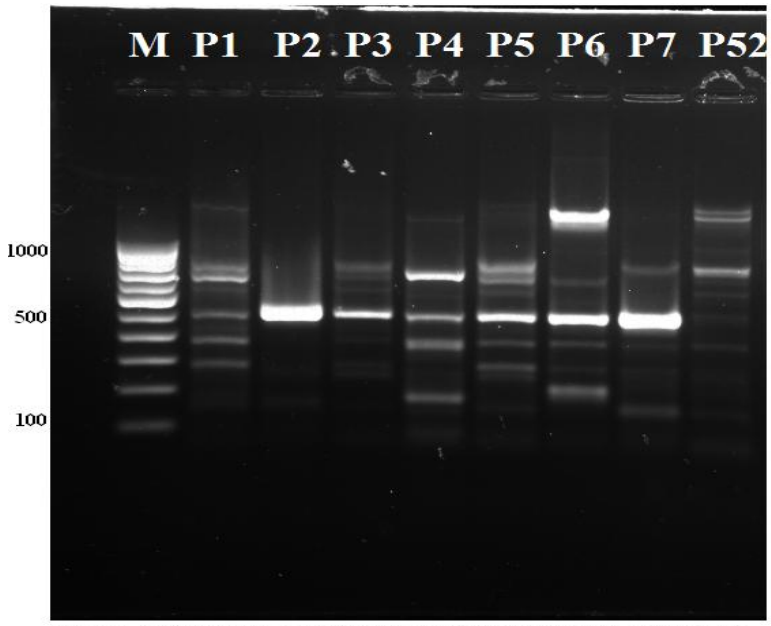


Figure 2 . ERIC PCR profiles. Lanes: M: Marker, 100 bp ladder; P1: isolate P1; P2: isolate P2; P3: isolate P3; P4: isolate P4; P5: isolate P5; P6:isolate P6; P7: isolate P7; P52: Vaccine strain.

Fig.3

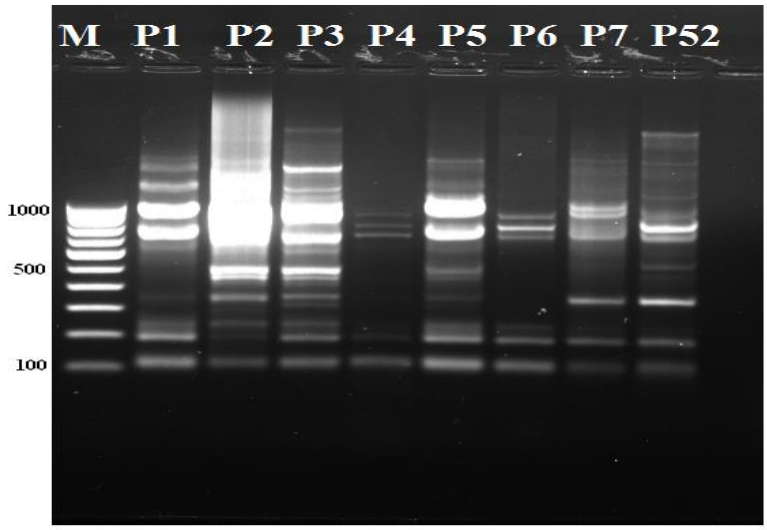
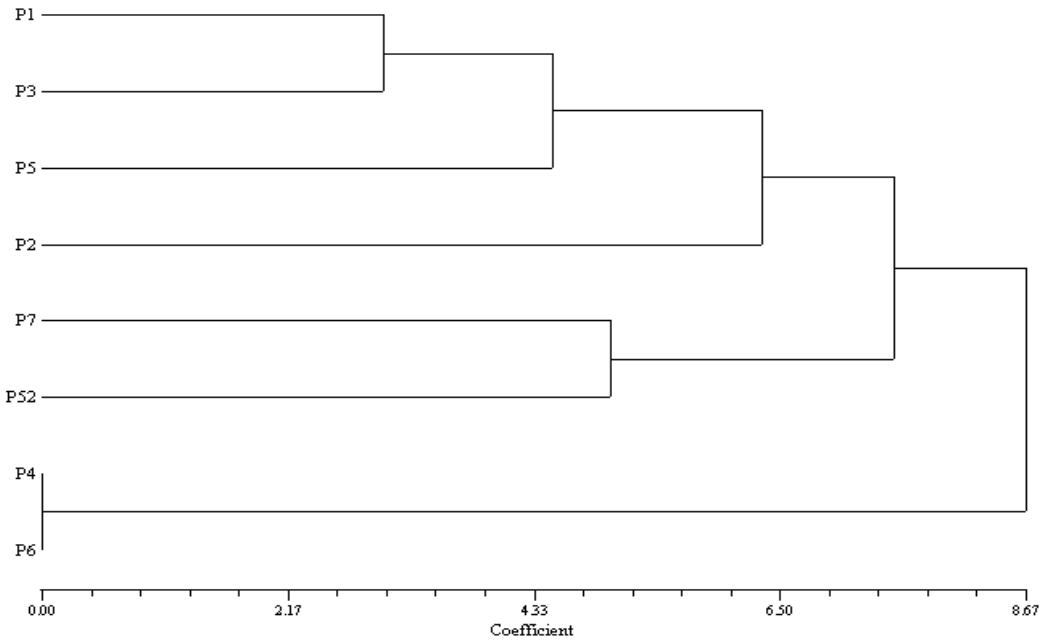


Figure 3. REP PCR profiles. Lanes: M: Marker, 100 bp ladder; P1: isolate P1; P2: isolate P2; P3: isolate P3; P4: isolate P4; P5: isolate P5; P6:isolate P6; P7: isolate P7; P52: Vaccine strain.

Fig.4 Dendogram of isolates of *P. multocida* based on combined ERIC and REP profiles.



The HSB-PCR method simplifies characterization of type B isolates by detecting all three HS associated serotypes in a single process. The versatility of this assay will enable the technique to be used for routine diagnosis in countries not currently able to perform conventional methods (Saxena *et al.*, 2002). In present study molecular methods were found to be of more efficient than conventional methods.

In combined molecular typing using data produced by ERIC and REP PCR D value of 0.95 was obtained.

Our findings indicate that in outbreak the isolates which were involved, were from different origin and genetic makeup. In dendogram it can visualized that the isolates of one location are more closely related. Similar findings have been reported from India (Biswas *et al.,* 2004) and other countries (Gunuwardana *et al.,* 2000) (Towensend *et al*., 1997).

Generally, oil adjuvant vaccine or alum-precipitated and aluminium hydroxide gel vaccines with a vaccine strain of *P. multocida* (P52) are currently used in India for control of HS. The variation at molecular level in the field isolates, in comparison to the vaccine strain, warrants an imminent strategy for controlling HS.

In conclusion, we have shown H.S. is still an important disease spreading in rainy season. PCR based screening can be used effectively for detection of HS-associated serotypes of *P. multocida* (B:2, B:5 or B:2,5). The present investigation indicates that genetic variation of isolates of same or different origin can be visualized by ERIC and REP-PCR.

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