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Rapid virulence typing of *Pasteurella multocida* in sheep isolates of Tamil Nadu

P PRABHAKAR[1], A THANGAVELU[2], T G PRABHAKAR[3], J JOHN KIRUBAHARAN[4] and N DANIEL JOY CHANDRAN[5]

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 051 India

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ABSTRACT

Pasteurellosis caused by *Pasteurella multocida* is an acute septicaemic disease characterized by high morbidity and mortality in cattle, sheep, goat and poultry resulting in severe economic losses. There are different methods of characterization of the *P. multocida* isolates like capsular and virulence typing. Capsular typing of isolates by PCR demonstrated 4 capsular types A(12), B(3), D(3) and F(2), respectively. A recently reported multiplex PCR based method was used to determine virulence genes, viz. *tbpA*, *pfhA*, *toxA* and *hgbB*. Among 14 virulent genes, 4 important virulence genes were detected by PCR. In the present study high prevalence of *pfhA* (100%), TbPA (95%) and low prevalence of *hgbB* (40%) and ToxA (30%) genes were found in sheep isolates. This simplified multiplex PCR method for rapid detection of 4 virulence genes may be used as epidemiological marker in *P. multocida* infection in different species of animals.

Key words: *Pasteurella multocida,* Pasteurellosis, Sheep, Virulence typing

Pasteurellosis caused by *Pasteurella multocida* is an acute septicaemic disease characterized by high morbidity and mortality in cattle, sheep, goats and poultry resulting in economic losses. Sheep pasteurellosis is one of the common infectious and economically important bacterial diseases recorded in temperate, subtropical areas (Chandrasekaran *et al*. 1991). Pasteurellosis in sheep is serotype specific and it is necessary to monitor continuously the prevalence and emergence of various serotypes to develop suitable prophylactic strategy (Rodger 1982).

A PCR based virulence genotyping method was suggested for epidemiological studies of *P. multocida*. Among 14 virulence associated genes present in *P. multocida* (Ewers *et al*. 2006), 4 important virulence genes were used in this study.

Two iron acquisitions related genes, *tbpA* and *hgbB* were associated to first and second mechanism of iron acquisition, respectively *phfA* has a role in bacterial adhesion and *toxA* produce dermonecrotoxin. *ToxA* showed association with isolates causing swine diseases, *TbPA* and pftA with isolates causing bovine diseases (Ewers *et al*. 2006). Association between *Pfha1*, *HgbB*, *TbPA* and *ToxA* genes and disease incidence in sheep till now remains unclear. Therefore, the present study was conducted on the prevalence and its association of *Pfha1*, *HgbB*, *TbPA* and *ToxA* genes in *P. multocida* sheep isolates.

Present address:[1]S R F (p_prabha2003@yahoo.co.in), [2,3,4] Professor (thangavelu_a@hotmail.com; jjohnk@ gmail.com; tgprabhakar@gmail.com), [5]Professor and Head (danieljoychandran @gmail.com), Department of Veterinary Microbiology, Madras Veterinary College, Chennai 600 007.

MATERIALS AND METHODS

Nasal swabs (359) from sheep exhibiting pneumonic symptoms like respiratory distress, profuse nasal discharge and sneezing were collected from all over Tamil Nadu (Coimbatore, Chennai, Chennai-Slaughter house, Rajapalayam, Thoothukudi, Tirunelveli, Nagarkoil, Vandalur, Kancheepuram) at different time intervals for the period between 2010–11.

Mouse bioassay: The specimens were pooled flock-wise of same area or village exhibiting similar symptoms and inoculated in brain heart infusion broth. After 16 h incubation at 37°C, 0.1 ml from each broth culture was injected subcutaneously into a set of swiss albino mice. Heart blood smears, aspirates of heart blood and impression smears of spleen, liver and lung were collected from dead mice.

Aspirated heart blood from dead mice was streaked onto 10 % sheep blood agar and incubated at 37°C. The heart blood was also inoculated in BHI broth and incubated at 37°C overnight and the broth culture was streaked onto blood agar and McConkey agar.

DNA extraction by high salt method: DNA was extracted from the cultures by high salt method as described by Senthilkumar and Ramadass (2000). Overnight culture was

centrifuged at 10 000 rpm for 20 min. The pellet was washed with phosphate buffered saline twice. The resulting pellet was suspended in 0.5 ml of solution I (10 mMTris HCl, 10 mM KCl, 10 mM $MgCl_2$,2 mM EDTA) and in 0.5 ml of solution II (10Mm Tris HCl, 10 mM KCl, 10 mM $MgCl_2$,2 mM EDTA,0.4M NaCl) and incubated at 37°C for 15 min in water bath; 50 micro litre of 10% SDS and 250µl of 6 M NaCl were then added and centrifuged at 10 000 rpm for 5 min at 4°C and ethanol precipitated. The pellet was resuspended in LTE (low tris EDTA) buffer and stored at –20°C until used.

P. multocida species specific PCR (PM-PCR): The species specific primers KMTIT7 and KMTISP6 designed by Townsend *et al.* (1998a) were used to amplify the gene sequences in *P.multocida.*

Capsular PCR typing: The *P. multocida* capsular serogroup primers designed by Townsend *et al.* (2001) were used for capsular PCR typing. The isolates were streaked into blood agar slants in duplicates and sent to Indian Veterinary Research Institute (IVRI) for serotyping.

Multiplex virulence PCR typing: The *P.multocida* virulence primers designed by Sina Atashpaz *et al.* (2009) were used for virulence PCR typing.

RESULTS AND DISCUSSION

Pasteurellosis caused by *P.multocida* is an opportunistic respiratory pathogen of sheep in tropical climate causing high morbidity and mortality. The predisposing factors include the hot tropical climate and stress induced by management practices such as docking, drenching, castration etc., as reported by Chandrasekaran *et al.* (1991)

A total of 359 samples suspected for pasteurellosis were pooled area-wise into groups and were subjected to biological test in mice and 20 isolates of *P.multocida* were obtained.

Mouse bio-assay revealed that 17 *P. mutocida* isolates were virulent with mean death time (MDT) between 12–18 h. Three isolates had a MDT between 19 and 24 h. The mouse bioassay findings are in agreement with the findings of Mustafa *et al.* (1978), Diallo *et al.* (1995) and Suresh babu (2003).

Heart blood smear, liver and spleen impression smear showed characteristic bipolar organism on Leishman staining and Gram negative coccobacilli by Gram staining method in accordance with Adlam and Rutter (1989).

The isolates showed typical cultural characteristics of dew drop, mucoid, non haemolytic colonies in blood agar. No growth was observed in McConkey agar. Grams staining of the smears revealed characteristic gram negative coccobacillary organisms. The isolates were positive for indole, nitrate reduction, oxidase and catalase. These results were same as that of Kawamota *et al.* (1990 and OIE (2004).

Pasteurella multocida species specific PCR (PM–PCR) assay developed by Townsend *et al.* (1998) was used in this study to identify the subspecies of *P.multocida* by amplifying 460 bp DNA fragment within KMTI gene using the primers KMTISP6 and KMTIT7. The molecular weight of the PCR

Table 1. PCR primers and cycling conditions

S.no	Gene	Primer sequence	Product size	PCR cycling conditions
1	*KMT1*	KMT1T7 ATCCGCTATTTACCCAGTGG KMT1SP6 GCTGTAAACGAACTCGCCAC	460 bp	94°C for 5 min 95°C for 30 sec 55°C for 30 sec 30 cycles 72°C for 30 sec. 72°C for 7 min.
2	*hyaD-hyaC*	CAPA-FWD TGCCAAAATCGCAGTCAG CAPA-REV TTGCCATCATTGTCAGTG	1044bp	
	bcbD	CAPB-FWD CATTTATCCAAGCTCCACC CAPB-REV GCCCGAGAGTTTCAATCC	760 bp	95°C for 5 min 95°C for 1 min
	dcbF	CAPD-FWD TTACAAAAGAAAGACTAGGAGCCC CAPD-REV CATCTACCCACTCAACCATATCAG	657 bp	55°C for 1 min. 30 cycles 72°C for 1 min
	ecbJ	CAPE-FWD TCCGCAGAAAATTATTGACTC CAPE-REV GCTTGCTGCTTGATTTTGTC	511 bp	72°C for 7 min
	fcbD	CAPF-FWD AATCGGAGAACGCAGAAATCAG CAPF-REV TTCCGCCGTCAATTACTCTG	851 bp	
3		ToxA F TCT TAG ATG AGC GAC AAG G	846 bp	
	toxA	ToxA R GAA TGC CAC ACC TCT ATA G		
		TbPA F TGG TTG GAA ACG GTA AAG C		94°C for 5 min
	tbPA	TbPA R TAA CGT GTA CGG AAA AGC C	728 bp	94°C for 45 sec
		HgbB F TC ATTG AGT ACG GCT TGA C		54°C for 50 sec 30 cycles
	hgbB	HgbB R CTT ACG TCA GTA ACA CTC G	499 bp	72°C for 50 sec.
		PfhA F AGC TGA TCA AGT GGT GAA C		72°C for 10 min
	pfhA	PfhA R TGG TAC ATT GGT GAA TGC TG	275 bp	

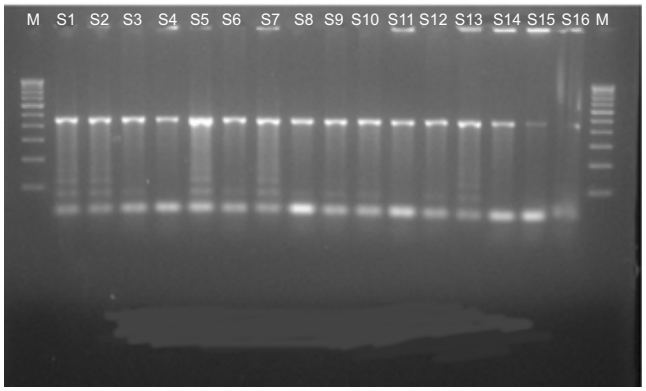

Fig. 1. *P. multocida* species specific PCR (PM-PCR)
S1, S16 P. multocida of sheep origin; M, 100 bp molecular weight marker

products of all the isolates were found indicating to be 460 bp, specificity for *P. multocida.* (Fig.1).

Capsular PCR method designed by Townsend *et al*. (2000) was used for capsular typing. Out of 20 isolates 12 belonged to 'A' serogroup, 3 to 'B' serogroup, 3 to 'D' serogroup and 2 to 'F' serogroup. These results also coincided with the serotyping results obtained from Indian Veterinary Research Institute (IVRI).

Virulence genes considered in this study have an important role in epidemiological studies and in pathogenesis of *P. multocida*. The role of iron in pathogenesis of *P. multocida* is important, so 2 iron acquisition related genes, *TbPA* and *HgbB*, genes were studied. In *P. multocida*, 2 independent non sidrophore-mediated iron acquisition mechanisms have been identified. The first mechanism involves iron-binding proteins expressed on the outer membrane of the bacterial cell, interacting directly with host iron-binding glycoproteins. The second mechanism includes bacterial proteins that bind haemoglobin and haemoglobin complexed to the host glycoprotein (Cox *et al.* 2003). Two iron acquisition related genes, *TbPA* and *HgbB*, studied in this paper are associated to first and second mechanism of iron acquisition respectively. The virulence gene *PfhA* has a role in bacterial adhesion and *toxA* produce dermonecrotoxin.

Ewers *et al*. (2006) reported that *ToxA* gene was associated with swine diseases. Jalal Shayegh *et al*. (2010) reported that *TbPA* gene and *pfhA* gene normally associates with bovine diseases. *TbPA* and *ToxA* genes were shown to be associated with ovine diseases as per Shayegh *et al*. (2008).

In this study a high prevalence of pfhA (100%) gene was noticed in *P. multocida* sheep isolates whereas Shayegh *et al*. (2010) reported that *pfhA* gene is highly prevalent among

Table 2.Virulence genes in different sheep isolates as per serogroup

Isolates	*PfhA*-Bacterial adhesion gene	*hgbB*-Iron acquisition gene	*tbpA*-Iron acquisition gene	*toxA* - dermonecrotoxin gene	Capsular serogroup
Sheep 1	+	-	+	-	'B'
Sheep 2	+	-	+	-	'B'
Sheep 3	+	-	+	-	'B'
Sheep 4	+	-	+	-	'A'
Sheep 5	+	+	+	-	'F'
Sheep 6	+	+	+	-	'F'
Sheep 7	+	-	+	-	'A'
Sheep 8	+	+	+	-	'F'
Sheep 9	+	-	+	-	' A'
Sheep 10	+	-	+	+	' A'
Sheep 11	+	-	+	+	' A'
Sheep 12	+	-	+	+	' A'
Sheep 13	+	+	-	-	' A'
Sheep 14	+	-	+	+	' D'
Sheep 15	+	-	+	-	'A'
Sheep 16	+	+	+	-	'A'
Sheep 17	+	+	+	-	'A'
Sheep 18	+	+	+	+	'A'
Sheep 19	+	-	+	-	'A'
Sheep 20	+	+	+	+	' D'

S. no	Virulence genes	Capsular 'A'	Capsular 'B'	Capsular 'D'	Capsular 'F'	Total %
1	*PfhA* - Bacterial adhesion gene	100%	100%	100%	100%	100
2	*hgbB* – Iron acquisition gene	33%	0%	50%	100%	40
3	*tbpA* – Iron acquisition gene	92%	100%	100%	100%	95
4	*toxA* –Dermonecrotoxin gene	33%	0%	100%	0%	30

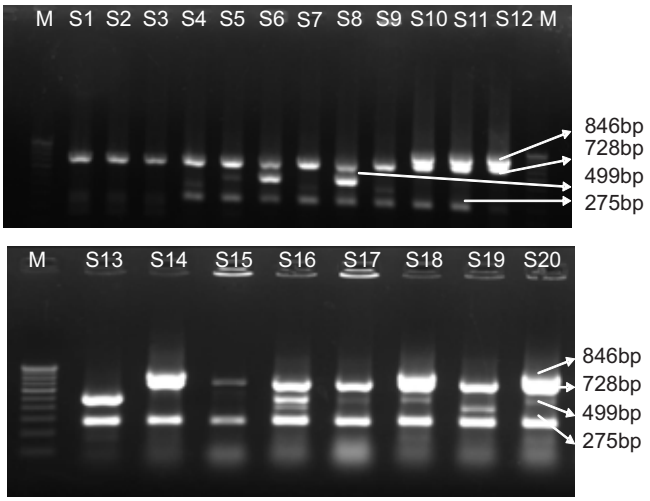

Fig. 1. Rapid virulence typing of *P. multocida* sheep isolates in Tamil Nadu.
S1, S20 P. multocida of sheep origin; M, 100 bp molecular weight marker

cattle isolates. *TbPA* gene is present in 95% of sheep isolates and this findings coincides with result of Shayegh *et al*. (2008), which says that *TbPA* gene, highly prevalent among *P. multocida* isolates causing respiratory infection in sheep. It was also reported that the presence of *TbPA* in bovine isolates of *P. multocida* normally associated with pneumonia and haemorrhagic septicemia (Veken *et al*. 1994, Ogunnariwo and Schryvers 2001).

Further present results confirmed the presence of *TbPA* gene (95%) in high numbers among sheep isolates in Tamil Nadu. There is a significant association between this *TbPA* gene and ovine disease (Shayegh *et al. 2008)*. Along with *TbPA* gene, *toxA* gene plays a major role in ovine disease as per the report of Shayegh *et al*. (2008). But the presence of *toxA* (30%) genes in sheep isolates was found to be much lower in the present study. The presence of another virulent gene *HgbB*(40%) in sheep was also found to be lower (Fig. 2; Table 2).

Presence of *TbPA* gene and pfhA genes in high percentage of sheep isolates and presence of ToxA gene in less percentage of sheep isolates indicate that there should be more studies in this aspect to select virulent genes for epidemiological studies, as this could be used as a simple and effective epidemiological tool for disease surveillance.

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