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Polymerase chain reaction typing of *Pasteurella multocida* capsules isolated in Iran

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Summary

Capsules from a range of pathogenic bacteria are the key determinants of virulency. The capsule has been implicated in virulence of *Pasteurella multocida*. In this study a type-specific polymerase chain reaction (PCR) assay was used for capsular typing of 39 avian *P. multocida* isolates from Iran. The PCR amplified a fragment of 1044 bp from all of tested isolates. It was found that all avian *P. multocida* isolates belonged to capsular type A. The sequence alignment of the fragment showed a high similarity (>96%) with the published sequences of *P. multocida hya* gene in the Gene Bank. It was recognised that *P. multocida* capsular group A is the dominant cause of fowl cholera in Iran.

Key words: *P. multocida*, Capsule, PCR, Typing

Introduction

Pasteurella multocida is a heterogenous species of Gram-negative bacteria and is a commensal of the upper respiratory tract of many animal species. However, under predisposing circumstances, the organism is the etiology of a wide range of economically important infection in domesticated animals. (Davies *et al*., 2003). *P. multocida* is known to cause fowl cholera, a widely distributed and economically important disease of poultry, particularly chickens, turkeys, ducks and geese. (Rhoades and Rimler, 1989; Rimler and Glisson, 1997). Many *P. multocida* strains express a polysaccharide capsules on their surface and isolates can be differentiated serologically by capsular antigens into serogroups A, B, D, E and F (Rimler and Rhoades, 1987). The disease caused by the organism is generally dependent on capsular type, since serogroups B and E cause haemorrhagic septicaemia in cattle and buffalo; serogroup A causes fowl cholera in poultry; and serogroup D causes atrophic rhinitis in pigs (Boyce and Adler, 2000). Until recently, little was known about the composition of capsular materials of *P. multocida* serogroups other than that of serogroup A, which is known to be sensitive to the action of hyaluronidase. It was demonstrated that the major polysaccharide component of the capsule in serogroup A was hyaluronic acid (Rosner *et al*., 1992). Hyaluronic acid is found in the capsules of other bacteria such as group A streptococci (Pruimboom *et al*., 1996). The capsular materials of serogroups D and F have been identified primarily through the action of mucopolysaccharidases. On the basis of decapsulation profiles of *P. multocida* by these enzymes, it was proposed that serogroups D and F produced capsular materials that contained heparin and chondrotin-sulfates, respectively. The monosaccharide analysis of a serogroup B *P. multocida* strain determined that the purified capsular polysaccharide was composed of arabinose, mannose, and galactose in a ratio of 0.5:2.0:0.8 (Townsend *et al*., 2001). The chemical composition of the serogroup E capsule still remains unknown.

Capsules have a significant role in determining access of certain molecules to the cell membrane, mediating adherence to surfaces, and increasing tolerance of desiccation (Nilsson *et al*., 1997; Boyce and

Adler, 2000). Survival of *P. multocida* outside the host and resistance to phagocytosis in the host are associated with the presence of capsule. In turkeys, the capsule seems to inhibit opsonization or create physical interferences of receptor-ligand binding between phagocytes and opsonized bacteria (Pruimboom *et al*., 1996). Experiments with purified *P. multocida* B:2 capsular extract have indicated that it has significant antiphagocytic activity (Munian-dy *et al*., 1992).

Conventionally, an indirect haemagglutination test (IHA) for typing of specific capsule antigens was described (Carter, 1955).

A limitation of capsule typing is the difficulty of inducing antibodies to specific antigens. It is especially difficult to prepare high titred antiserum against serogroups A, D and F strains. This difficulty may be due in part to the presence of inert capsule materials such as hyaluronic acid and mucopolysaccharide (Carter and Rundell, 1975). To overcome the difficulties with IHA test, non-serologic tests were designed for recognition of types A (Carter and Rundell, 1975), D (Carter and Subronto, 1973) and F (Rimler, 1994).

Recently, a polymerase chain reaction (PCR) assay has been developed for capsular typing of *P. multocida* strains. The identification and sequence analysis of the biosynthetic locus of the capsule *P. multocida* organisms provided a greater understanding of its capsular polysaccharide composition and the genetic basis for the serologic differences. This assay represented a rapid and reproducible alternative to the serologic and non-serologic methods (Townsend *et al*., 2001). The objective of the present study was to identify the capsular type(s) of avian *P. multocida* isolates by PCR assay.

Materials and Methods

Bacterial strains and growth conditions

Thirty-nine avian isolates of *P. multocida* were used in this study. Some phenotypic and molecular characters of 25 of this isolates have been described previously (Jabbari *et al*., 2002).

Also, 14 isolates were kindly provided by Dr. M. Banani, Razi Vaccine and Serum Research Institute, (RVSRI), Karadj, Iran. All of these isolates were recovered from fowl cholera cases in RVSRI.

The capsular reference strains including X-73 (capsular type A) and M1404 (capsular type B) were provided by Dr. M. Vasfi Marandi (Faculty of Veterinary Medicine, University of Tehran, Tehran); the P3881 strain (capsular type D) was obtained from Dr. G. Guanawardana (Veterinary Research Institute, Seri Lanka). The isolates were stored at -70°C in fresh sheep blood. Stock bacterial cultures were streaked onto blood agar and incubated overnight at 37°C.

DNA extraction

Chromosomal DNA was prepared according to Wilson *et al*., (1995) with some modifications. The cultures were initially grown on 5% sheep blood agar plates with incubation at 37°C for 18 to 24 hrs. Growth from a single colony was inoculated into a test tube containing 3 ml brain heart infusion broth (BHI) and incubated at 37°C for 18 to 24 hrs with a little rotation to get approximately 10^8 cells/ml.

One and half-ml aliquots of the BHI were centrifuged at 13,000 × g for 5 min. The supernatant was discarded and the pellets were washed twice in PBS at 13,000 × g for 5 min. The bacterial cell pellets were resuspended in 600 µl natrium tris-EDTA (NTE) buffer (pH = 7.4). Three µl of a 20 mg/ml stock solution of proteinase K (GibcoBRL) was added to the bacterial suspensions to a final concentration of 100 µg/ml. The bacterial suspensions were then incubated at 37°C overnight in the presence of 0.5% SDS. The cell lysate was extracted with one volume of ultrapure phenol (GibcoBRL) previously saturated with tris (pH = 8.3). The mixture was rotated vigorously for 5 min at room temperature and then centrifuged at 13,000 × g for 10 min. The upper phase was collected without disrupting the interface and transferred to a clean 1.5-ml microtube. This phase was mixed with one volume of a phenol/ chloroform/isoamylalcohol (24:24:1) mixture and centrifuged at 13,000 × g for 5 min.

The extraction with phenol/chloroform /isoamylalcohol was repeated until there was no visible material at the interface between the aqueous and organic phase. The DNA was precipitated by the addition of sodium acetate and absolute ethanol. The DNA was then washed with 70% ethanol, dried at room temperature and resuspended in TE buffer (pH = 8). The DNA was stored at -20°C until used. The absorbency of DNA was measured at 260 nm in a spectrophotometer (Sambrook *et al.*, 1989).

Primers

Oligonucleotide primers were based on specific sequences in each capsular group and designed to generate PCR product of unique size (Townsend *et al.*, 2001). The primer sequences used in this study are listed in Table 1.

PCR conditions

PCR conditions was followed as described previously (Townsend *et al.*, 2001) with some modifications. The PCR amplification mixture (25 µl) was contained each primer at a concentration of 3.2 µm, each deoxynucleoside triphosphate at a concentration of 200 µm, 1 × PCR buffer, 2 mM $MgCl_2$, and 0.5 U of Taq DNA polymerase. Twenty ng of template *P. multocida* genomic DNA was added to the mixture. All amplifications were performed with the Ependorf PCR system.

Amplification was performed for 30 cycles. The cycles include an initial denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The final cycle was followed by an extension at 72°C for 5 min.

The amplified products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The PCR products of selected isolates were purified and sequenced.

Results

PCR amplification of type specific capsular fragment of the isolates produced a single 1044-bp band, corresponding to the reference strain type A (Fig. 1). Fragments of 760 and 650 bp were amplified with *bcbD* and *dcbF* specific primers using types B and D reference strains. PCR produced a fragment of 760 bp using the bovine *P. multocida* isolate. This isolate is used for preparation of haemorrhagic septicaemia vaccine in Iran. Fig. 2 shows the PCR products of different capsular groups.

The sequence alignment of the fragments were compared with the previous sequences in Gene Bank, showed 96–99% similarity (Table 2). The accession number of the cap loci sequences of Iranian isolates are as follows: AY225344, AY225343, AY225345, AY225342, AY157572, AY225347, AY225346, AY225345.

Discussion

There are a number of difficulties associated with conventional capsular serotyping of *P. multocida*. A particular problem inherent in serological typing and diagnosis of *P. multocida* is the inagglutinability of serogroups A, D and F in homologous antisera.

Inagglutinability of capsulated *P. multocida* is associated with a serologically inert

Table 1: sequences of the oligonucleotides used in the *P. multocida* capsular multiplex PCR typing assay

Capsule type	Gene	Name	Sequence	Amplimer size (bp)
A	*hyaD*	CAPA-FWD	TGCCAAAATCGCAGTCAG	1044
		CAPA-REW	TTGCCATCATTGTCAGTG	
B	*bcbD*	CAPB-FWD	CATTTATCCAAGCTCCACC	760
		CAPB-REW	GCCCGAGAGTTTCAATCC	
D	*dcbF*	CAPD-FWD	TTACAAAAGAAAGACTAGGAGCCC	650
		CAPD-REW	CATCTACCCACTCAACCATATCAG	
All	*kmt1*	KMT1T7	ATCCGCGATTTACCCAGTGG	460
		KMTSP6	GCTGTAAACGAACTCGCCAC	

Table 2: Comparison of nucleotide sequence of the PCR product of *P. multocida* isolate PMI030 (local vaccinal strain) with other *P. multocida* presence in the Gene Bank

Gene	Strain	Country	Accession No.	Size	Identity (%)
kmt1	PMI030	Iran	AY157572	416	100
kmt1	PMD	Srei Lanka	AY225342	429	99
kmt1	PMB2	Iran	AY225341	447	99
kmt1	PM70	USA	AE006174	422	98
kmt1	PMI035	Iran	AY225343	425	98
kmt1	PM0113	Australia	AF016259	418	98
kmt1	PMI047	Iran	AY225344	462	97
hyaD	PMA	Australia	AF067175	968	99
hyaD	PMI035	Iran	AY225345	864	99
hyaD	PMI047	Iran	AY225347	907	96
hyaD	PMUS	USA	AF036004	746	99
hyaD	P4676	USA	AE006116	668	96

Fig. 1: Multiplex PCR to determine capsular group of *P. multocida*. Lane 1-6: the results of PCR reaction with DNA from representative avian *P. multocida* isolates. Lane 7: result of PCR with DNA from bovine *P. multocida.* Lane 8: DNA size marker

Fig. 2: Optimization of PCR for capsular typing of *P. multocida*. Lane 1: molecular DNA marker. Lanes 2-4: PCR products from reference strains M1404 (type B, 760 bp), X-73 (type A, 1044 bp) and P3881 (type D, 650 bp), respectively. Lane 5: the *P. multocida* specific fragment (*kmt1*, 460 bp)

substance that is a component of the bacterial capsule. In *P. multocida* serogroup A this substance is hyaluronic acid (Rimler, 1994). By the same reason, it is especially difficult to prepare high-titered antisera against serogroups A and D specific antigens.

The nucleotide sequence of the *P. multocida* capsule biosynthetic locus has been studied. The genes in the capsular biosynthetic loci, were grouped into three functional regions. Regions 1 and 3 contain a total of six genes, which are involved in transport of the polysaccharide while region 2 contains four to nine genes, which are involved in the biosynthesis of the polysaccharides (Boyce and Adler, 2000). Sequence alignments of genes in the regions 1 and 3 are highly similar among all *P. multocida* capsular types, whereas, the region 2 contains the hypervariable fragments. Comparative analysis of this region among different capsular groups revealed that sequences within *hyaD, bcbD, dcbF, ecbJ* and *fcbD* were highly specific for their respective serogroups.

An alternative and highly specific capsular multiplex PCR assay that is based on nucleotide sequence variation within the five capsular biosynthetic loci was developed. The primer sequences used in capsular PCR typing have been designed to generate a sero-group specific fragment with unique size. This method provides a rapid and highly specific alternative to conventional capsular serotyping (Townsend *et al*., 2001). The capsular PCR typing has been used for determining the capsular types of large numbers of avian, ovine and porcine *P. multocida* strains (Davies *et al*., 2003). This method was also found to be extremely useful for determining the capsular types of bovine *P. multocida* isolates (Davies *et al*., 2004).

So far, four capsular serogroups (A, B, D and F) are recognized among strains of avian *P. multocida* (Rhoades and Rimler, 1987). However, strains of serogroup A are recognised as the primary and dominant cause of fowl cholera, whereas isolates of serogroups B, D and F are less frequently associated with the disease (Wilson *et al*., 1993).

According to results of this study, all avian *P. multocida* isolated from Iran belonged to capsular type A. Sotoodehnia *et al*., (1986) reported one serotype B and one serotype D avian *P. multocida* isolate from Iran. Somatic serotyping of avian *P. multocida* isolates originated from northern provinces of Iran has been studied previously. Somatic serotypes 1, 3, 3 × 4 and 4 have been reported among these isolats (Jabbari *et al*., 2001).

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