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Identification of The Capsule Type of Pasteurella Multocida Isolates from Cases of Fowl Cholera by Multiplex PCR and Comparison with Phenotypic Methods

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ABSTRACT

The ability of *Pasteurella multocida* to invade and multiply in its host is enhanced by the presence of the capsule, one of the most important virulence factors for this bacterium. Capsular typing methods are often used in epidemiological and pathogenesis studies of this agent. Five different serogroups have been identified based on serological typing. However, such tests are laborious, and agglutination of homologous antiserum may fail. The aim of this study was to develop a multiplex PCR protocol for the identification of the *hyaD-hyaC* and *dcbF* genes specific to serogroups A and D, respectively, and to compare these results with those of phenotypic tests for 54 strains isolated from fowl cholera cases in southern Brazil. The kappa coefficient and chi-square statistics were calculated to assess the agreement between the diagnostic methods and to determine the significance of the results, respectively. The multiplex PCR was able to detect the evaluated genes. Forty-nine strains (90.74%) were classified into serogroup A, and only two isolates (3.7%) were not identified as belonging to any of the serogroups analyzed. In contrast, with the phenotypic tests, only 41 strains (75.93%) were classified into serogroup A and 11 samples (20.37%) were unidentifiable. Of the strains analyzed, 70.37% were classified into the same serogroup (A) by both methods, and the kappa coefficient (k = 0.017) indicated poor agreement between the tests. Thus, multiplex PCR is an alternative for *P. multocida* capsular typing, as it allows the simultaneous and rapid detection of genes and also provides a greater strain-typing capacity.

INTRODUCTION

Members of the family *Pasteurellaceae* are implicated in a number of diseases, many of which with respiratory signs (Nascimento *et al*., 2009). One member of this family, *Pasteurella multocida*, is the causative agent of multiple different diseases that have great economic impact on animal production, including hemorrhagic septicemia in cattle, atrophic rhinitis in swine and fowl cholera (FC) in domesticated and wild birds (Harper *et al*., 2006; Glisson, 2008; Rigobelo *et al*., 2013).

The ability of *P. multocida* to invade and multiply within the host is enhanced by the presence of its capsule, a polysaccharide structure that is one of the most important virulence factors for this species (Wilkie *et al.*, 2012). Functions assigned to the capsule include desiccation resistance, antiphagocytic activity and interaction with the complement system (Boyce *et al*., 2000). Additionally, there are conflicting reports in the literature regarding the possible role of capsule in the adhesion to host cells and tissues (Harper *et al.*, 2012). The importance of the capsule in *P. multocida* adherence possibly depends on its strain and host cell type (Pruimboom *et al*., 1996).

All isolates can be classified into five different capsular types or serogroups (A, B, D, E and F) according to the presence of capsular antigens (Harper *et al*., 2012). Most cases of FC are caused by serogroup A and, more rarely, by types F and D (Dziva *et al*., 2008). There is a recognized and documented association between capsule type and particular hosts and diseases (Harper *et al*., 2012). The possible interrelationship between capsular type, pathogenesis and host predisposition to a particular serogroup has been suggested (Chung *et al*., 1998). However, the molecular and cellular bases for these host and disease associations remain unknown (Harper *et al*., 2012). Typing methods are used to study the pathogenesis and epidemiology of *P. multocida* as well as to investigate the diversity of isolates from different hosts (Christensen & Bisgaard, 2006).

The serogroups are usually identified with the passive hemagglutination test (Carter, 1955). However, other phenotypic and non-serologic tests have been proposed due to both the time required to perform the serologic test and the need for specific antiserum for each type of capsule. Furthermore, there is the possibility of agglutination failure with homologous antiserum (Davies *et al*., 2004; Arumugam *et al*., 2011). Certain serotypes of *P. multocida* exhibit distinctive features, which have been exploited for their rapid identification. For instance, serogroup A can be easily identified by cross-streaking with hyaluronidase-producing *Staphylococcus aureus* that depolymerizes the hyaluronic acid found in encapsulated type A *P. multocida* strains (Carter & Rundell, 1975). Serotype D strains typically produce a coarse flocculation via an unknown mechanism when acriflavine dye is added to a broth overnight culture (Carter & Subronto, 1973). Additionally, the elucidation of the genetic basis for capsule biosynthesis in more recent years has facilitated the development of a method for laboratory typing *P. multocida* isolates with a multiplex PCR assay based on specific gene sequences for each capsular type (Townsend *et al*., 2001).

The objectives of this study were to develop a multiplex PCR protocol for the identification of capsular genes specific to serogroups A and D in strains of *P. multocida* isolated from FC cases and to compare these results with non-serologic phenotypic tests.

MATERIALS AND METHODS

Selected strains and DNA extraction

A total of 54 strains of *P. multocida* isolated from clinical cases of FC in southern Brazil were selected. The reference strains of *P. multocida* ATCC 15742 (capsular type A), ATCC 12945 (capsular type A), ATCC 12946 (capsular type B) and a toxigenic clinical sample (capsular type D) were selected as positive controls for multiplex-PCR and phenotypic tests. All samples were stored in sheep blood at a temperature of -70°C. Reactivation and preliminary tests for the confirmation of pure samples of *P. multocida* were performed according to Glisson *et al*. (2008). The isolates were reactivated in brain heart infusion broth (BHI - Oxoid; Cambridge, United Kingdom) and incubated at 37°C for 24 hours. After this period, the isolates were plated on blood agar base (Oxoid; Cambridge, United Kingdom) supplemented with 5% (defibrinated sheep blood) and on MacConkey agar (Oxoid; Cambridge, United Kingdom) where the bacterium rarely grows, to differentiate of other members of the *Pasteurellaceae* family that can grow on this agar (Glisson *et al.*, 2008). The morphology of colonies grown in blood agar were evaluated. Catalase and oxidase tests were conducted in addition to Giemsa staining to observe the characteristics of the bacterial bipolar cells. A 1-mL aliquot of a BHI overnight culture for each sample was prepared to extract DNA using the NucleoSpin Tissue commercial kit (Macherey Nagel; Düren, Germany). Prior to multiplex PCR typing, a PCR protocol for species-specific amplification of the *kmt* gene was performed, as described by Townsend *et al*. (1998).

Table 1 - The capsule genes selected, the processes in which they are involved, primer sequences and amplicon sizes.

Gene	Process or enzyme involved	Primer sequence (5'-3')	Amplicon (bp)	Reference
hyaD-hyaC	Capsule type A biosynthesis	TGCCAAAATCGCAGTCAG TTGCCATCATTGTCAGTG	1044	Townsend *et al.* (2001)
bcbD	Capsule type B biosynthesis	CATTTATCCAAGCTCCACC GCCCGAGAGTTTCAATCC	760	Townsend *et al.* (2001)
dcbF	Capsule type D biosynthesis	TTACAAAAGAAAGACTAGGAGCCC CATCTACCCACTCAACCATATCAG	657	Townsend *et al.* (2001)

Multiplex PCR protocol

The multiplex PCR protocol proposed to detect capsular genes was established based on Townsend *et al*. (2001). In addition to the genes *hyaD-hyaC* and *dcbF*, a multiplex PCR protocol was performed to detect *bcbD*, a gene specific to serogroup B. The sequence of each primer pair and the expected size of each amplicon are described in Table 1. Briefly, the PCR mix consisted of 2.5 µL of 10X PCR buffer, 1.25 µL of 2.5 mM deoxynucleoside triphosphates, 2 µL of each primer pair at 20 pmol (Invitrogen; Carlsbad, USA), 2 U of Taq DNA polymerase (Centro de Biotecnologia UFRGS; Porto Alegre, Brazil), 1.25 µL of 2.5 mM $MgCl_2$, 5 µL of DNA and sterile ultrapure water for a final volume of 25 µL. Amplification was performed in a Swift MaxPro thermal cycler (ESCO Technologies, Singapore) under the following reaction conditions: initial denaturation (95°C for 3 minutes) followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds) and elongation (72°C for 60 seconds) and a final elongation stage (72°C for 10 minutes). Electrophoresis of the amplified products was carried out in a 1% agarose gel (Invitrogen™, Carlsbad, USA) stained with ethidium bromide, and the amplified products were visualized in an ultraviolet light transilluminator (Pharmacia LKB MacroVue; Uppsala, Sweden). The negative controls were selected among different members of the *Pasteurellaceae* family (*Reimerella anatipestier* ATCC 11845, *Mannheimia haemolytica* ATCC 29694, *Bordetella avium* ATCC 35086 and *Pasteurella gallinarum* ATCC 13360). Lastly, PCR mix without the addition of extracted DNA was used as a negative control for the reaction.

Phenotypic tests

Two aliquots of a BHI overnight culture were selected for the identification of serogroup D strains with the acriflavine test and for identification of serogroup A strains with the hyaluronidase test, according to Carter & Subronto (1973) and Carter & Rundell (1975), respectively. For the acriflavine test, 2 mL of a BHI overnight culture was centrifuged at 6.000 rpm for 20 minutes, and 0.5 mL of acriflavine solution diluted at 1:1000 (Sigma Aldrich; Saint Louis, USA) was added to 0.5 mL of the concentrated culture obtained. The isolates that exhibited flocculation after an interval of 5 minutes were assigned to serogroup D (Figure 1). In order to identify serogroup A with the hyaluronidase test, strains were plated on blood agar (Oxoid; Cambridge, United Kingdom) in lines approximately 5 mm apart. Next, a *Staphylococcus aureus* strain that produces the hyaluronidase enzyme was streaked at a 90° angle to the *P. multocida* lines. The plates were incubated at 37°C for 24 hours. As hyaluronic acid is the main chemical component of the capsular type A structure, those colonies exhibiting growth inhibition in close proximity to the *S. aureus* streak were assigned to serogroup A (Figure 1).

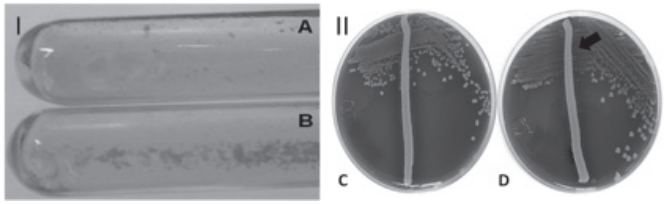

Figure 1 – I - Acriflavine test: (A) negative reaction. (B) Positive reaction between the inoculum of *P. multocida* type D and acriflavine solution. II - Hyaluronic acid test: (C) colonies of *P. multocida* lack growth inhibition in the vicinity of the staphylococcal colony. (D) Colonies of *P. multocida* capsular type A (arrow).

STATISTICAL ANALYSIS

Data were statistically analyzed using the software program Statistical Package for Social Sciences, (SPSS Inc., Chicago, USA). Kappa coefficients and chi-square (X^2) statistics were calculated to assess agreement and to determine the significance of the results, respectively. p-value <0.05 was considered statistically significant.

RESULTS

The multiplex PCR protocol was able to detect the indicated genes of interest (Figure 2). The results of capsular typing indicated that there was a significant difference between the tests (Table 2). Using multiplex PCR, 49 isolates (90.74%) were classified into serogroup A and two

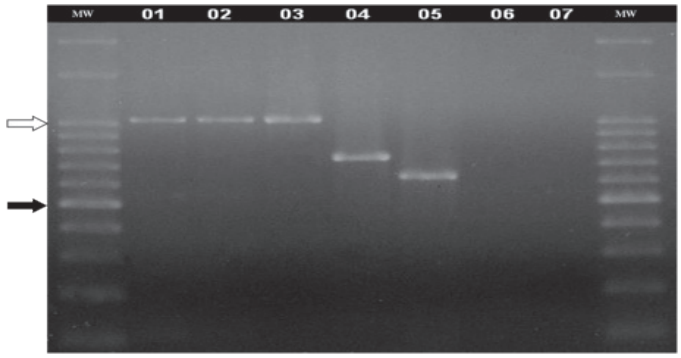

Figure 2 – Electrophoresis on a 1% agarose gel stained with ethidium bromide and the amplification products associated with the studied genes (multiplex PCR): *hyaD-hyaC* (1044 bp), *bcbD* (760 bp), *dcbF* (657 bp). Legend: MW = molecular weight marker (100 bp), 01 and 02 = samples; 03 = *P. multocida* ATCC 12945; 04 = *P. multocida* ATCC 12946; 5 = *P. multocida* toxigenic; 6 = *M. haemolytica* ATCC 29694; 7 = negative control for the reaction. The filled arrow indicates a 500-bp fragment; the white arrow indicates a 1000-bp fragment.

Table 2 - Capsular typing of 54 *Pasteurella multocida* strains of avian origin with phenotypic tests and multiplex PCR.

Analysis	Serogroup			
	A (%)	D* (%)	U** (%)	Total (%)
Phenotypic test	41[a] (75.93)	2[a] (3.7)	11[a] (20.37)	54 (100)
Multiplex PCR	49[b] (90.74)	3[a] (5.56)	2[b] (3.7)	54 (100)

Different letters in the same column represent significant differences, X2 (1) = 4.267/ p=0.034 ($p < 0.05$)

**Untypeable

isolates (3.7%) were not identified by the test. In contrast, only 41 samples (75.93%) were assigned to type A using the phenotypic test, whereas 11 samples (20.37%) were unidentifiable (Table 2). There was no significant difference in the number of capsular type D strains classified between the two tests. In terms of percent agreement, 70.37% of the strains were classified into the same serogroup by both the phenotypic tests and multiplex PCR (Table 3). However, the kappa coefficient (k = 0.017) indicated a poor agreement between the two methods (Landis & Koch**,** 1977). This fact is explained by the high frequency of serogroup A among the strains analyzed.

Table 3 - Correlation of capsular typing results between phenotypic tests and multiplex PCR.

		Multiplex PCR			
	Serogroup	A	D	U**	Total
Phenotypic tests	A	37	3	1	41
	D	2	0	0	2
	U**	10	0	1	11
	Total	49	3	2	54

Percent agreement: 70.37%
Kappa coefficient: 0.017 (0.062-0.212)
**Untypeable

DISCUSSION

The capsule is the main virulence factor identified for *P. multocida*, and a possible relationship between capsular type, pathogenesis and host susceptibility to a particular serogroup has been suggested (Chung *et al*., 1998). Serogroup A was identified in 49 of 54 isolates by multiplex PCR. These results are consistent with the findings of Leotta *et al*. (2006), who obtained similar results with 8 of 9 strains isolated from poultry in Argentina. Similarly, Shivachandra *et al*. (2006) identified 92 strains as belonging to type A from among 94 samples isolated from chickens in India. Jabbari *et al*. (2006) classified all of their 35 Iranian samples in the same serogroup. Type A is the main serogroup found in avian isolates (Rhoades & Rimler, 1989). In this study, only 3 isolates were found to belong to serogroup D. This serogroup is considered rare (Glisson, 2008). Davies *et al*. (2003) determined that only 8% of samples isolated in birds belonged to type B, 5% belonged to serogroup D and 4% belonged to type F.

Two strains were not identified with the multiplex PCR protocol adapted from Townsend *et al*. (2001). The authors of that study determined that 2-5% of strains were classified as untypeable. In other similar studies, 2-9% of samples were not classified (Dziva *et al*., 2004; Jamaludin *et al*., 2005, Leotta *et al*., 2006). In this case, potential analysis could involve evaluation for the presence of a non-capsulated strain by electron microscopy, as one isolate was not identified by non-serological tests (Davies *et al*., 2003). Another possibility may be that these strains belong to serogroup F, which was not analyzed in the current work.

A comparison of non-conventional serological tests and molecular methods for determining the capsular type revealed important variations in typing capability. The reduced capacity to determine the capsular type with conventional methods was also observed in other studies (Shivachandra *et al*., 2006; Arumugan *et al*., 2011). Since the development of a PCR technique for typing, this molecular method has been considered by some authors to be the gold standard technique for capsular typing, replacing phenotypic tests, particularly the passive hemagglutination test (Dziva *et al*., 2008). The agglutination failure of serogroups A, D and F with homologous antisera is one of the main causes of reduced sensitivity in this phenotypic test (Jabbari *et al*., 2006). Another important issue is that the passive hemagglutination test developed by Carter (1955) can be rendered ineffective by the loss of *P. multocida* capsule after repeated subcultures *in vitro* (Dziva *et al*., 2008). In the study by Shivachandra *et al*. (2006) comparing both methods, 16% of 123 isolates from different avian species were not identified by conventional tests, whereas all samples were typed by multiplex PCR. Similarly, Arumugan *et al*. (2011) found that 48% of strains were non-typeable strains with the hyaluronidase and acriflavine test or through the use of specific antisera to identify the capsule types A, D and B, respectively.

Diverging classifications for these two methodologies have been noted in other studies (Ewers *et al*., 2006;

Arumugan *et al*., 2011). The chemical similarity of polysaccharides, which comprise the capsule, may interfere with specificity of phenotypic tests (Ewers *et al*., 2006).

The difficulty in obtaining capsular type-specific antisera as well as the necessity of identifying and typing field isolates in the early stages of infection or prior to the development of an efficient homologous vaccine for an FC challenge are important justifications for use of the molecular method (Shivachandra *et al*. 2006; Dziva *et al*., 2008). Moreover, multiplex assay protocols allow for the simultaneous detection of different genes, reducing the amount of reagents and the time required to obtain results (Perry *et al*., 2007).

In addition to the specific relationships between *P. multocida* serogroups and particular diseases and species, there is also a dominant geographical distribution of capsular types (Zaglic *et al*., 2005). However, these relationships have exhibited variations in recent years. Examples include the increase in the number of pig pneumonia cases caused by capsular type D; this disease was formerly associated with serogroup A (Borowski *et al*., 2007). Another example is the spread of hemorrhagic septicemia cases in cattle associated with serogroup B, which was previously concentrated only in Southeast Asia (Khan *et al*., 2011). Likewise, type F, which was initially associated with avian species, has been identified in other hosts, including pigs and rabbits that presented respiratory diseases (Davies *et al*., 2004; Jaglic *et al*., 2004). Future studies with larger numbers of isolates from FC cases in different regions of the country may also exhibit this variation. It remains unclear whether a particular detection system may be associated with disease, host or population structure. Therefore, the use of phenotypic tests and confirmatory genotypic techniques remains crucial in establishing a definitive diagnosis of *P. multocida* infection. Finally, multiplex PCR is an alternative to comparative phenotypic tests for the identification of capsular *P. multocida* because it allows for the simultaneous, rapid detection of genes and provides a greater capacity for strain typing.

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