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Capsular serotyping of *Pasteurella multocida* from various animal hosts – a comparison of phenotypic and genotypic methods

Arumugam, N.D.[1], Ajam, N.[1], Blackall, P.J.[2], Asiah, N.M.[3], Ramlan, M.[4], Maria, J.[4], Yuslan, S.[5] and Thong, K.L.[1*]

[1] Microbiology Division, Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

[2] Animal Research Institute, Department of Primary Industries and Fisheries, Yeerongpilly, Queensland, Australia

[3] Diagnostic & Quality Assurance Division, Department of Veterinary Services, 62630 Putrajaya, Malaysia

[4] Veterinary Research Institute, Jalan Sultan Azlan Shah Utara, 31400 Ipoh, Perak, Malaysia

[5] Seksyen Pembangunan Komoditi Ternakan, Jabatan Perkhidmatan Veterinar Negeri Selangor, Lot 2, Jalan Utas 15/7, 40630 Shah Alam, Malaysia.

* Corresponding author email: thongkl@um.edu.my

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Abstract. One hundred and fourteen strains of *Pasteurella multocida* were isolated from different domestic animals species (cattle, buffalo, sheep, goat, pig, rabbit, dog, cat), avian species (chicken, duck, turkey) and wild animals (deer, tiger, orang utan, marmoset). The serogroups of *P. multocida* were determined by both conventional capsular serotyping and a multiplex PCR assay targeting specific capsular genes. Based on the conventional serotyping method, the 114 strains of *P. multocida* were subtyped into 55 species-specific (untypeable strains) *P. multocida*, 15 serogroup A, 23 serogroup B and 21 serogroup D. Based on the multiplex PCR assay on the specific capsular genes associated with each serogroup, the 114 strains were further divided to 22 species-specific *P. multocida* (KMT1 – 460 bp), 53 serogroup A (A – 1,044 bp), 33 serogroup B (B – 760 bp) and 6 serogroup D (D – 657 bp). No serogroup E (511 bp) or F (851 bp) was detected among the Malaysian *P. multocida*. PCR-based typing was more discriminative and could further subtype the previously untypeable strains. Overall, there was a significant and positive correlation between both methods in serogrouping *P. multocida* ($r = 0.7935$; $p < 0.4893$). Various serogroups of *P. multocida* were present among the livestock with 75% of the strains belonging to serogroups A or B. PCR serotyping was therefore a highly species-specific, sensitive and robust method for detection and differentiation of *P. multocida* serogroups compared to conventional serotyping. To the best of our knowledge, this is the first report from Malaysia of the application of a PCR to rapidly define the species-specific *P. multocida* and its serogroups as an important zoonotic pathogen in Malaysia.

INTRODUCTION

The Gram negative bacterium *Pasteurella multocida* infects a wide range of animal species, causing diseases such as atrophic rhinitis in pigs (Chanter & Rutter, 1989), fowl cholera in poultry (Glisson *et al.*, 2003) and haemorrhagic septicaemia and shipping fever in cattle (Carter & De Alwis, 1989; Frank, 1989). Carter (1955) first reported type-specific capsular antigen in *P. multocida*. Later, Rimler & Rhoades (1989) indicated there are five distinct capsular serogroups of *P. multocida* (serogroups A, B, D, E and F). Fowl cholera of birds is caused by serogroup A and, to a lesser extent serogroup D (Rhoades & Rimler, 1989). In pigs, serogroups A and D are associated with pneumonia and atrophic rhinitis, the latter condition being

associated with toxigenic strains (Chanter & Rutter, 1989). In cattle, serogroup A is commonly associated with pneumonia (Frank, 1989) while serogroups B and E are associated with haemorrhagic septicaemia (Carter & De Alwis, 1989).

In Malaysia, hemorrhagic septicemia (HS) caused by *P. multocida* serogroup B is one of the most important diseases of cattle and buffaloes. For example, in Terengganu, Malaysia, an outbreak of HS caused the deaths of 187 cows and buffaloes worth more than RM200,000 (http://archives.foodsafety.ksu.edu/animalnet/2000/2-2000/an-02-14-00-01.txt). Similarly, pasteurellosis was one of the common diseases encountered in ducks in Malaysia causing severe economic losses. In 2004, fowl cholera caused 50% of the mortalities seen in 400 populations of Muscovy ducks in the district of Kuala Krai, Kelantan, Malaysia (2005 Annual report of Regional Veterinary Laboratory, Kota Bharu – unpublished report).

The linkage between certain disease conditions or hosts and the capsular serogroup as described above indicates the importance of accurate identification of the capsular serogroups of *P. multocida* isolates. Unfortunately, the original methodology developed for the identification of the capsular serogroup that is the indirect haemagglutination test (Carter, 1955), is technically difficult and time consuming to perform (Rimler & Rhoades, 1989). Hence, the non-serological tests for serogroups A and D, a capsule depolymerisation test in the presence of hyaluronidase-producing *Staphylococcus aureus* for serogroup A (Carter & Rundell, 1975) and a characteristic floccular reaction with acriflavine for serogroup D (Carter & Subronto, 1973), have been widely adopted by veterinary diagnostic laboratories, particularly in developing countries. For serogroups B and E, alternative serological methodologies such as counter-immunoelectrophoresis (Carter & Chengappa, 1981) and co-agglutination test (Rimler, 1978) have been developed. Townsend *et al*. (2001) reported a PCR-based method for the capsular typing of *P. multocida*. The capsular PCR is now regarded as the gold standard test (Dziva *et al*., 2008) and has been used in a number of studies of isolates from a range of animal hosts (Jaglic *et al*., 2005; Varga *et al*., 2007). While the advantage of the PCR-based approach has been well recognized, the adoption of PCR-based technologies in veterinary laboratories in the developing world has been limited in some areas by factors such as cost and technical expertise. In Malaysia, capsular typing of *P. multocida* has been done by conventional serological or non-serological methods. Due to the limitation of the conventional serological method, the objective of this study was to apply a multiplex PCR to detect the capsular genes of *P. multocida* and to differentiate the serogroups in different animal hosts.

MATERIALS AND METHODS

Bacterial strains

A total of 114 strains of *P. multocida* were isolated from animal hosts over a period that covered 1996 to 2004 (Table 1). All of these strains were previously isolated and identified at the Bacteriology Unit of Veterinary Research Institute, Ipoh, Perak and the Regional Veterinary Laboratory Petaling Jaya, Malaysia. The identification was based on phenotypic properties.

Confirmed strains of *P. multocida* capsular serogroups A (ATCC 11039), B (M-1404), D (P3881), E (P1234) and F (P4679) were provided by the Regional Veterinary Laboratory Petaling Jaya as positive control strains.

Conventional capsular typing of *P. multocida*

All the *P. multocida* strains were capsular typed by the methods established by the Malaysian Veterinary Research Institute, the national reference center for zoonotic diseases. For the identification of serogroup A, the *P. multocida* strains were streaked transversely across a whole brain heart infusion (BHI) agar plate to provide lines of growth approximately 3-5 mm

apart. Then, a hyaluronidase-producing strain of *Staphylococcus aureus* was streaked heavily at 90° angles to the *P. multocida* strains and the plates were then incubated overnight at 37°C. If there was a diminution in the size of the *P. multocida* colonies in the region adjacent to the *Staphylococcus* streak, the isolate was assigned to serogroup A. For serogroup B, antiserum produced from rabbits (provided by Malaysian Veterinary Research Institute) was used. A drop of saline (0.85% NaCl) was placed onto a clean grease-free glass slide and the isolate emulsified onto it. A drop of type B antiserum was added and mixed well using a loop. The occurrence of agglutination indicates a positive reaction and the isolate was assigned to serogroup B. Serogroup D was determined by the characteristic flocculation with acriflavine neutral (0.1%, Sigma USA.). Approximately, 3 ml of overnight cell culture in BHI broth was harvested by centrifugation at 268 x *g*. A 2.5 ml volume of the supernatant was discarded and 0.5 ml of aqueous solution of acriflavine neutral was added to the remaining 0.5 ml of broth culture. The suspension was mixed well by pipeting and transferred to a narrow Kahn tube (12 mm x 75 mm). The cell suspension was allowed to settle at room temperature for 15 minutes. If an isolate gave a flocculation at the bottom of the Kahn tube, then the isolate was assigned to serogroup D.

Capsular typing via Polymerase Chain Reaction (PCR)

The detection of capsular genes by PCR for all serogroups was done according to the method described by Townsend *et al.* (2001) with some modifications. An overnight cell culture grown in Brain Heart Infusion (BHI) broth was harvested by centrifugation at 11,337 x *g* for 5 min. The cell pellet was resuspended in 100 µl of TE (10 mM Tris; 1 mM EDTA, pH 8.0) buffer and boiled for 10 min, followed by immediate chilling. The cell lysate was centrifuged at 11,337 x *g* for 5 min and the supernatant was used as the DNA template. The concentration of the DNA template was determined using a spectrophotometer at $OD_{260/280nm}$ (Eppendorf, Germany). The oligonucleotide sequences of primers previously published by Townsend *et al.* (2001) were synthesized by Operon (Germany).

A multiplex-PCR was used to confirm the strains as *P. multocida* and to determine if the strains were of serogroups A, B or D. For this multiplex PCR, each reaction mixture contained 50 ng of DNA template, 1 X PCR Buffer, 280 µM dNTPs mix, 2.7 mM $MgCl_2$ buffer and 1.5 U of *Taq* DNA polymerase (Promega, USA). The primer concentrations used were: primers KMT1T7, KMT1SP6 (*P. multocida*) and A-FWD, A-REV (serogroup A) 3.2 µM, primers B-FWD, B-REV (serogroup B) 1 µM and primers D-FWD, D-REV (serogroup D) 1.2 µM (Operon, Germany). Separate singleplex PCRs for serogroups E and F were performed on all strains. The reaction mixture of these two single PCR assays was the same as the multiplex PCR with the relevant primer concentrations being 3.2 µM.

PCR conditions consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation (95°C, 45s), annealing (56.1°C, 45s), extension (72°C, 1 min), and a final extension step of 72°C for 5 min. As a positive control, equal amounts of 100 ng DNA template from each of the reference strains for serogroups A, B and D were mixed and 1 µl of the mixture was used for every PCR run. A negative control (without DNA template) was also included in every run.

Statistical Analysis

Correlations were evaluated using the Pearson's correlation test. For all statistical comparisons, the level of significance was set at $p < 0.05$.

RESULTS

A total of 114 strains of *P. multocida* were obtained from different animal hosts (bovine, porcine, poultry, avian) and from different locations during the period from

1996 to 2004 (Table 1). During the period indicated, there was an increase of 25% in the number of isolation. From 1996 to 2000, isolation of *P. multocida* was relatively rare compared to the years after 2000. Most of these strains were isolated from chickens (24% - 2001), buffaloes (21% - 2003 and 2004), cattle (17% - 2002), ducks (13% - 2003 and 2004), goats (7% - 2003) and pigs (7% - 2003).

Certain animal hosts predominated in certain years, as indicated by the results in Table 2. In 2001, 30% (8/27) of *P. multocida* were isolated from chickens, 42% (8/19) from cattle in 2002, 29% (7/24) from buffaloes in both 2003 and 2004 and 26% (7/27) from chickens in 2004. Since 2002, *P. multocida* were also isolated from more wild animal hosts such as deer, tigers, marmosets and orang-utan (primates). Among all the implicated animal hosts, the avian and bovine animals each contributed 38% respectively to the total isolation of *P. multocida* during the study period (1996-2004).

Based on conventional serotyping, the distribution of *P. multocida* is as follows: 15 strains of serogroup A, 23 strains of serogroup B and 21 strains of serogroup D. The other 55 strains were classified as untypeable as they showed negative results in the three conventional serotyping tests (Table 2).

The multiplex PCR was successfully optimized and applied to confirm the serogroups of *P. multocida*. The reference strains of *P. multocida* serogroup A, B and D yielded the expected results in the multiplex PCR – all three strains of different serogroups gave the 460 bp band specific for *P. multocida*. The serogroup A strain gave the 1,044 bp band characteristic of this serogroup. The serogroup B reference strain gave the 760 bp band while the serogroup D reference strain gave the 657 bp band which are the expected results (Figure 1). The reference strains of *P. multocida* serogroups E (P1234) and F (P4679) gave the expected amplicons of 511 bp and 851 bp, respectively as indicated in Figure 2.

All 114 strains were confirmed as *P. multocida* by the PCR method and they all yielded the species-specific 460 bp with the KMT1T7 and KMT1SP6 primers (Figure 1).

Based on PCR analysis, there were 53 strains of serogroup A, 33 strains of serogroup B, 6 strains of serogroup D and 22 strains which gave no band other than the species-specific 460 bp band and were therefore classified as untypeable (Table 2). No field isolate gave any amplification with either the serogroup E or F.

Table 3 directly compares the results of the conventional and genotypic capsular typing methods. The PCR method was able to recognize an additional 10 strains of serogroup B as compared with the conventional method. For these 10 additional serogroup B strains, the host species were goat (one strain), deer (one isolate), cattle (two strains), buffalo (two strains) and chicken (four strains). While the conventional method assigned 21 strains to serogroup D, only 3 were confirmed as serogroup D by PCR; the majority of the strains were re-assigned to serogroup A. Of the 55 strains that could not be assigned to any serogroup by the conventional method, 23 were re-assigned to serogroup A, 10 to serogroup B and two to serogroup D by the PCR method. A correlation analysis between the conventional capsular serotyping system and the multiplex PCR assay showed a significant and positive correlation ($r = 0.7935$; $p < 0.4893$) between the respective methods.

The results of PCR capsular typing indicated that the major serogroup in ducks, chickens, cattle, and pigs was serogroup A while serogroup B was the dominant serogroup in buffaloes. Only PCR typing approach recognized the presence of capsular serogroup B strains from a goat, a deer and four chickens.

DISCUSSION

There has been an increase of 25% in the isolation rate of *P. multocida* strains from

Table 1. Distribution of *P. multocida* strains from different animal hosts in Malaysia (1996 to 2004)

Year	Animal Hosts															Total
	Cattle	Buffaloes	Sheep	Goat	Pig	Rabbit	Dog	Cat	Chicken	Duck	Turkey	Deer	Tiger	Orang-utan	Marmo-set	
1996					1											1
1997																0
1998		2														2
1999		3							1	1						5
2000	1	4		1					5			1				12
2001	4		1	1	1				8			1				16
2002	8	1		1	2	1	1		1	4		1	1	1		22
2003	3	7		5	2				5	5						27
2004	3	7		0	2			1	7	5	1	2			1	29
Total	19	24	1	8	8	1	1	1	27	15	1	5	1	1	1	114

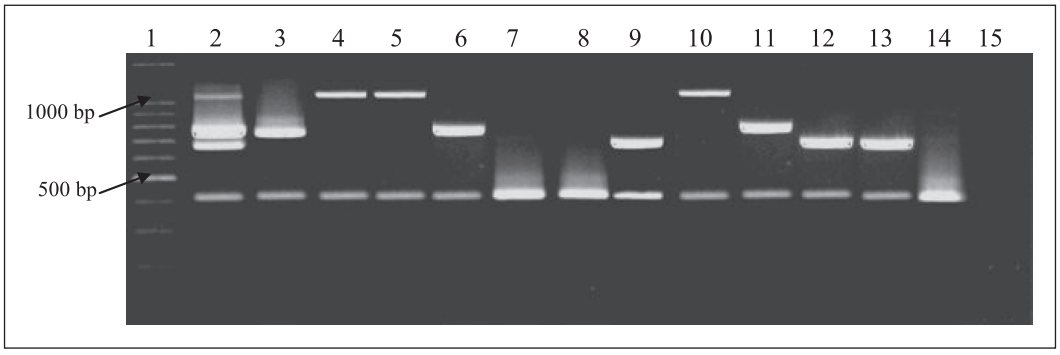

Figure 1. Multiplex PCR Profiles of representative *P. multocida* strains

Lanes: 1, Marker, 100 bp DNA ladder; 2, Positive control (containing DNA templates of reference serogroups); 3, 710/00B (760 bp & 460 bp – *P. multocida*, serogroup B); 4, 44/01A (1,044 bp & 460 bp – *P. multocida*, serogroup A); 5, 7760/03A (1,044 bp & 460 bp – *P. multocida*, serogroup A); 6, 5893/02B (760 bp & 460 bp – *P. multocida*, serogroup B); 7, 2557/00U (460 bp – *P. multocida*, untypeable strain); 8, 3466/02U (460 bp – *P. multocida*, untypeable strain); 9, 10377/03D (657 bp & 460 bp – *P. multocida*, serogroup D); 10, 2006/04A (1,044 bp & 460 bp – *P. multocida*, serogroup A); 11, 9/04B (760 bp & 460 bp – *P. multocida*, serogroup B); 12, 2910/02D (657 bp & 460 bp – *P. multocida*, serogroup D); 13, 7855/02D (657 bp & 460 bp – *P. multocida*, serogroup D); 14, 5650/02U (460 bp – *P. multocida*, untypeable strain); 15, Negative control (water as template)

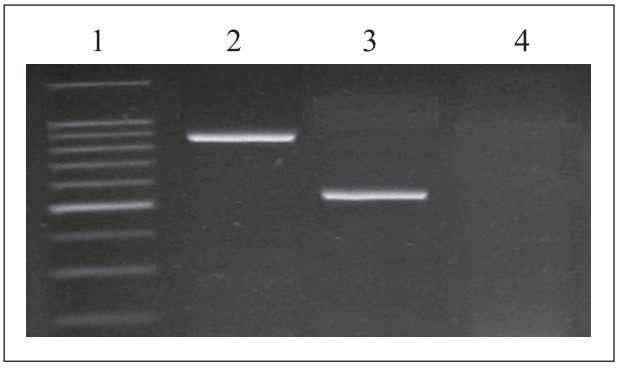

Figure 2. PCR Profiles of *P. multocida*, serogroup E and F strains (reference strains)

Lanes: 1, Marker, 100 bp DNA ladder; 2, Reference strain P1234 (511 bp – *P. multocida*, serogroup E); 3, Reference strain P4679 (851 bp – *P. multocida*, serogroup F); 4, Negative control

Table 2. Distribution of different capsular serogroups of 114 *P. multocida* strains from animals as determined by conventional and PCR- based methods

Animal Hosts	*P. multocida* and its serogroups determined by conventional serotyping					*P. multocida* and its serogroups determined by PCR serotyping				
	A	B	D	U[a]	TOTAL	A	B	D	U[a]	TOTAL
Goat	0	0	3	5	8	3	1	4	0	8
Duck	5	0	4	6	15	12	0	0	3	15
Chicken	3	0	7	17	27	17	4	0	6	27
Cattle	4	4	3	8	19	10	6	1	2	19
Pig	1	0	2	5	8	5	0	0	3	8
Buffaloes	1	19	1	3	24	3	21	0	0	24
Turkey	1	0	0	0	1	0	0	0	1	1
Rabbit	0	0	0	1	1	0	0	0	1	1
Tiger	0	0	1	0	1	0	0	1	0	1
Deer	0	0	0	5	5	0	1	0	4	5
Sheep	0	0	0	1	1	0	0	0	1	1
Dog	0	0	0	1	1	1	0	0	0	1
Primates	0	0	0	1	1	1	0	0	0	1
Cat	0	0	0	1	1	1	0	0	0	1
Marmoset	0	0	0	1	1	0	0	0	1	1
Total	15	23	21	55	114	53	33	6	22	114

[a] U = Untypeable strains

Table 3. Comparison of the conventional and genotypic capsular typing methods for 114 *P. multocida* strains

Conventional Result[a]	Genotypic Result			
	A	B	D	U[b]
A (15)	12	0	1	2
B (23)	0	23	0	0
D (21)	18	0	3	0
U[b] (55)	23	10	2	20
Total (114)	53	33	6	22

[a] Number in brackets is the number of strains assigned to this capsular serogroup by the conventional method

[b] U = Untypeable strains

the years 1996 to 2004. The increase rate was more obvious in years 2000 – 2004 (11% - 25%) (Table 1). The reasons for this increase could be that there was a closer monitoring by the national veterinary institutes and also an increase in awareness of this disease. Distribution of serogroups by multiplex PCR in animals was determined in a broad host range : serogroup A was found in goats (38%), ducks (80%), chickens (63%), cattle (53%) and buffaloes (13%); serogroup B in chickens (15%), cattle (32%) and buffaloes (88%); serogroup D in goats (50%) and untypeable strains in ducks (20%), chickens (22%), cattle (11%), pigs (38%), turkeys (100%), rabbits (100%), deer (80%), sheep (100%) and marmosets (100%). Therefore, in this study, different serogroups of *P. multocida* were found in a range of animal hosts and there was no obvious host specificity.

From the year 2000 onwards, *P. multocida* strains were also isolated from wild animals. Among the wild animals, serogroup D was found in tigers (100%), untypeable strains in marmosets (100%) and serogroup A in primates (100%). For house pets, strains of serogroup A were isolated from dogs (100%) and cats (100%).

In this study, the multiplex PCR of Townsend *et al.* (2001) was applied to perform capsular serotyping of 114 strains of *P. multocida* from Malaysia. Some modifications of the original methodology as described by Townsend *et al.* (2001)

were made. Firstly, for preparation of DNA template, a single bacterial colony was suspended in 100 µl of TE buffer and boiled, instead of transferring the colony directly into the PCR mixture as reported in Townsend *et al.* (2001). This gave a better background and less smearing (data not shown). Primer concentrations for B-FWD and B-REV were reduced to 1 µM while the D-FWD and D-REV primers were reduced to 1.2 µM from the 3.2 µM concentration reported by Townsend *et al.* (2001). Finally, the duration of the denaturation and annealing cycles was increased by 15 seconds each from the conditions used by Townsend *et al.* (2001). The modified multiplex PCR gave the expected amplicons size with the reference strains of serogroups A, B and D. Minor modifications to existing PCR assays have been used in other studies. Biswas *et al.* (2004) successfully used a modified version of the original *P. multocida* specific PCR of Townsend *et al.* (1998) for identification of serogroup B in haemorrhagic septicaemia.

As the capsular groups of greatest interest to veterinary laboratories in Malaysia are serogroups A, B and D, a structured approach was employed in the PCR analysis. All strains were initially tested with the multiplex PCR that involved identification at the species level and then assignment to serogroups A, B and D. All strains were tested in single assays for serogroups E and F, regardless of the serogroup result in the initial PCR. In a diagnostic situation, it is likely that only strains that were negative for serogroups A, B or D would be further examined by the serogroup E and F assays.

There have been few direct comparisons of the conventional non-serological methods of identifying *P. multocida* capsular serogroups A and D with the PCR-based method of Townsend *et al.* (2001). This study has shown that both the non-serological methods for serogroup A and D tests have major limitations. The hyaluronidase test for serogroup A failed to recognize 41 serogroup A strains as defined by PCR (Table 3). The acriflavine flocculation test for serogroup D wrongly assigned 18 strains to serogroup D. These particular 18 strains were then shown to be of serogroup A by PCR assay (Table 3).

The use of antisera to recognize serogroup B strains worked well for strains from cattle and buffalo. However, a total of 10 strains shown to be serogroup B by PCR failed to react with the serogroup B antiserum used in the current study. Of particular note was the finding that all chicken and deer strains of serogroup B (Table 2) were not recognized by the serological test.

Isolates of serogroup B are not commonly reported from avian hosts (Rhoades & Rimler, 1989). Jonas *et al.* (2001) have reported that one of nine isolates examined from fowl cholera outbreaks in Indonesia were serogroup B. In this study, four of the 27 chicken strains were shown to be serogroup B (Table 2).

A notable feature of the current study was the number of untypeable strains (22 of 114 – 19%) which could not be assigned to any capsular serogroup. A number of studies have reported the presence of untypeable strains using the modified Townsend *et al.* (2001) PCR method. These previous studies have reported levels of between 2% to 9% of isolates as untypeable (Davies *et al.*, 2003a, 2003b; Jamaludin *et al.*, 2005; Ewers *et al.*, 2006).

There is limited information on association of serogroups of *P. multocida* with infections in wild animals in Malaysia. This study has confirmed the presence of a range of serogroups of *P. multocida* in wild animals - tiger (serogroup D) and orang-utan (serogroup A) (Table 2). Further studies on larger numbers of strains from this group of animals need to be undertaken before any firm conclusions about the association of certain serogroups with wild animal hosts can be reached.

This study has shown that the non-serological methods of assigning strains of *P. multocida* to serogroups A and D are highly unreliable. Improvement in the specificity and sensitivity of serotyping *P. multocida* can be enhanced by adopting the

multiplex PCR of Townsend *et al.* (2001) to complement the conventional method.

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