Capsular types, LPS genotypes, and virulence-associated genes of *Pasteurella multocida* strains isolated from pigs¹⁾

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Summary

Pasteurella multocida (*P. multocida*), an opportunistic zoonotic pathogen associated with high morbidity and mortality in livestock, shows significant temporal and geographical variation in its serotype distributions and phenotypic characteristics. The aim of this study was To investigate capsular types, lipopolysaccharide (LPS) genotypes, and virulence-associated genes of *P. multocida* strains isolated from pigs. A total of 801 samples (lungs, tonsils, nasal swabs) were collected from slaughterhouses and various regions of the Hunan province. *P. multocida* strains were isolated from various samples, classified, and virulence-associated genes were detected by polymerase chain reaction (PCR). 124 *P. multocida* strains were assigned to six groups based on both capsular type and LPS genotype, namely A: L3 (capsular type A and LPS genotype 3, 64/124); A: L6 (16/124); D: L6 (38/124); F: L3 (4/124); L3 (1/124) and 1 untypable strain. Of the 23 virulence-associated genes investigated in this study, 14 were highly expressed in 98% to 100% of the 124 strains. While *tbpA* was undetectable in any of the isolated strains, *hsf-1*, *pfhA*, *tadD*, *toxA*, *pmHAS*, *hgbA*, *hgbB*, and *nanB* showed differential distribution among the strain groups. Interestingly, *pfhA* (Mutation or inactivation of *pfhA* was reported to decrease the virulence of *P. multocida*) was found in 46% of group A: L3 strains and in 100% of group F: L3 strains, but not found in other groups. Further investigation is needed to determine whether strains in group A: L3 show greater virulence than the A: L6 *P. multocida* strains.

Keywords: capsular types, LPS genotypes, *P. multocida*, virulence-associated genes

Pasteurella multocida (*P. multocida*) is a highly infectious pathogen capable of co-infecting poorly maintained livestock with other pathogens like *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, and porcine *Circovirus*, resulting in severe respiratory diseases or atrophic rhinitis in the swine industry(4, 6, 15, 17, 21, 22). The serotype distributions and phenotypic characteristics of *P. multocida* show both temporal and geographical variations. In 1952, Carter (5) classified *P. multocida* into five capsular serotypes (A, B, D, E and F) by indirect haemagglutination test. In 2001, Townsend et al. found that the capsular genotypes of *P. multocida* strains identified by mPCR were agreeable to Carter's

serotyping results. In 1972, Heddlestone and Gallagher (14) classified *P. multocida* strains into serotypes 1 through 16 using an agar-gel diffusion precipitin test based on lipopolysaccharide (LPS). LPS of *P. multocida* lacking the O antigen, contains a highly conserved inner core and a highly diverse outer core (13). In 2015, Harper et al. reported at least 22 types of outer core in *P. multocida*, that diversities in LPS were not outlined fully in Heddlestone's classification (12, 14). Harper et al. designed a rapid LPS-mPCR assay to genotype *P. multocida* strains based on the LPS outer core biosynthesis locus, and classified the strains into eight LPS genotypes: namely LPS genotype L1 covering Heddlestone's serotype 1 and 14; L2 covering Heddlestone's serotype 2/5; L3 covering Heddlestone's serotype 3 and 4; L4 covering Heddlestone's serotype 6 and 7; L5 covering Heddlestone's serotype 9; L6

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covering Heddlestone's serotype 10, 11, 12 and 15; L7 covering Heddlestone's serotype 8 and 13; and L8 covering Heddlestone's serotype 16 (13).

Capsule and LPS, along with other virulence factors such as adherence proteins (*ptfA*, *fimA*, *hsf-1*, *hsf-2*, *pfhA* and *tadD*), toxin (*toxA*), iron-intake related proteins (*exbB*, *exbD*, *tonB*, *hgbA*, *hgbB* and *Fur*), Sialic acid kinase (*nanB* and *nanH*), hyaluronic acid (*pmHAS*), superoxide dismutase (*sodA*, *sodC* and *tbpA*) and outer membrane proteins (*ompA*, *ompH*, *oma87* and *plpB*) constitute the virulence factors of *P. multocida* essential for causing acute disease-conditions (11, 18). These factors could help *P. multocida* escape the host immune-surveillance, and help the organism colonize the host-tissue, and elicit an inflammatory response (11, 16). Previous studies have reported *toxA* gene association with *P. multocida* serotype D strains; filament like heamoagglutinin *pfhA* gene association with *P. multocida* strains of serotypes A, B, E and F; and iron-take associated gene *tbpA* association with *P. multocida* strains of serotypes A and B (7).

In this study, tissue and swab samples collected in Hunan (China) between the years 2014 and 2016 were analyzed for *P. multocida* strains. The genotypes of the isolated *P. multocida* strains were characterized by using Townsend's mPCR methods specific for capsule typing (24) and Harper's LPS-mPCR (13). Additionally, 23 virulence-associated genes besides capsule and LPS of *P. multocida* were investigated using PCR methods previously described by Khamesipour et al. (18), and the distribution of these genes among different genotype groups were determined.

Material and methods

Samples and the reference strain collection. A total of 801 samples were collected between April 2014 and December 2016. Of these, 421 lung and 180 tonsil samples were collected from large slaughter houses in the Hunan province while another 147 lung samples and 53 nasal swabs from pigs with symptoms of fever, cough, and/or dyspnea were submitted from various regions of the Hunan province. The standard types A: 1 (capsular serotype A and LPS serotype 1, LPS genotype L1) *P. multocida* strain CVCC444 (*pmHAS*, *plpB*, *tadD* were confirmed positive by PCR and sequencing), type B: 2,5 (capsular serotype B and LPS serotype 2,5, LPS genotype L2) *P. multocida* strain CVCC434, and type D: 3 (LPS genotype L3) *P. multocida* strain CVCC439 (*hgbB*, *hsf-1*, *nanB* were confirmed positive by PCR and sequencing) were provided by Beijing Bei Na Chuang Lian Bio-tech Research Institute; the standard types B: 1 (capsular serotype B and LPS serotype 1, LPS genotype L1) *P. multocida* strain CVCC44-1 were provided by China Institute of Veterinary Drug Control (*ptfA*, *fimA*, *hsf-2*, *pfhA*, *exbB*, *exbD*, *tonB*, *hgbA*, *Fur*, *nanH*, *sodA*, *sodC*, *ompA*, *ompH*, *oma87* were confirmed positive by PCR and sequencing). The reference strain capsular type F was isolated and identified by the veterinary laboratory of the Hunan Agricultural University, China (GenBank accession number: KX161714); the reference strain LPS genotype 6

containing *toxA* gene was isolated and identified by the veterinary laboratory of the Hunan Agricultural University, China (Identification of LPS genotype, GenBank accession number: MH730062. Detection of *toxA* gene, GenBank accession number: MH730063).

Isolation and culture of *P. multocida*. For tissue samples, small amounts of tonsil or lung tissue (lung from slaughterhouse pigs or sick pigs) were cut from original samples under sterile conditions and weighed before grinding thoroughly. Grinding was performed with a ball mill (MM400Retsch GmbH, Haan, Germany) for 4 min at a frequency of 30 Hz. The homogenates were diluted with 0.9% normal saline (volume: 10 × tissue sample weight) and 20 µl of the diluted homogenates of each sample were inoculated on Blood Agar Base Medium (Barrett Biotechnology (Zhengzhou) Co. LTD, Henan, China), incubated at 37°C for 24 hrs. Nasal swabs were streaked onto blood agar plates followed by incubation at 37°C for 24 hrs. All of the inoculated plates were examined for colony formation.

Genomic DNA extraction. Three colonies phenotypically resembling *P. multocida* were selected from each inoculated blood agar plate and observed by smear microscopy. The colonies with the typical morphologic characteristics of *P. multocida* were enriched and preserved in glycerol and stored at -80°C. One representative colony from each tissue sample was picked for genome extraction and used subsequently as a DNA template for PCRs conducted in this study. The genomes from reference strains were simultaneously extracted and used as positive controls. Total DNA of bacterial strains was prepared with Bacteria Genomic DNA Extraction Kit (Takara Biomedical Technology, Beijing, China) following manufacturer's instructions.

Identification of *P. multocida* isolates and determination of capsular type. Primers for genus and capsular classification, described by Townsend (24), were synthesized by Shanghai Bo Shang Biotechnology Co. Ltd. *P. multocida*-specific primer KMT1 (460 bp) was used to identify *P. multocida* strains from the colonies with the typical morphologic characteristics of *P. multocida*; while primers A (1044 bp), B (760 bp), D (657 bp), E (511 bp) and F (851 bp), specific for each of the types of *P. multocida* capsule described by Townsend were used in the PCR systems for identification of *P. multocida* strain capsular types. Each PCR reaction sample-mix consisted of 25 µl of 2 × Taq Plus Master Mix (TransGen Biotech, Beijing, China), mixed with 22 µl of sterile water, 1 µl of each of the forward and reverse primers and 1 µl of DNA template. The PCR conditions consisted of a 5 min denaturation step at 94°C, followed by 30 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and elongating at 72°C for 45 sec, completed with a final extension step at 72°C for 7 min. PCR products were analyzed by electrophoresis on 1% agarose gel.

Identification of LPS genotypes of *P. multocida* strains. The LPS-mPCR method described by Harper et al. (13) was used for LPS genotype identification from genome templates of the selected colonies. Each reaction sample-mix contained 25 µl of 2 × Taq Plus Master Mix (TransGen Biotech, Beijing, China), mixed with 22 µl of sterile water, 1 µl of each of the forward and reverse primers and 1 µl of

DNA template. The PCR conditions consisted of a 5 min pre-denaturation step at 94°C, followed by 30 thermal cycles of denaturing at 94°C for 30 sec, annealing at 52°C for 30 sec and elongation at 72°C for 45 sec, followed by a final extension step at 72°C for 7 min. PCR products were visualized by electrophoresis on 2% agarose gel.

Detection of virulence-associated genes. 23 virulence-associated genes were detected by PCR, as described by Khamesipour (18). In brief, each PCR reaction-mix (total volume = 50 µl) consisted of 2 × Taq Plus Master Mix (TransGen Biotech, Beijing, China) 25 µl, mixed with sterile water 22 µl, 1 µl of each of the forward and reverse primers and DNA template 1 µl. The PCR and gel electrophoresis conditions were the same as that used for strain determination as described above.

Statistical analysis. Differences in *P. multocida* positive rates were investigated by chi-squared tests using SPSS software version 19.0 (IBM Inc., Chicago, IL, USA), and the results were considered significant when $P < 0.05$.

Results and discussion

Isolation of *P. multocida*. Of the 124 *P. multocida* isolates from 801 samples (described in detail in Table 1), lung samples of slaughterhouse pigs showed higher positive rates (11.9%) when compared to those from submitted diseased pigs (8.2%). The tonsil tissue samples of slaughterhouse pigs showed the highest positive rates (23.3%) compared to any of the other tissue sources, and were significantly higher than lung samples of slaughterhouse pigs ($P < 0.05$).

Identification of capsular types of *P. multocida* strain. As shown in Table 1, 80 isolates were of capsular type A, 38 of capsular type D, four of F and two untypable by our typing method while capsular types B, E were not found. Of the 50 isolates from slaughtered pig lungs, the positive rate of capsular type A was the highest ($P < 0.05$) with 49 isolates of capsular type A and one of F. For strains from tonsils of slaughtered pigs, 23 strains were of capsular type A, 27 of D and three of capsular type F. There were no significant differences between the positive rates of capsular type A and of D ($P > 0.05$), but the positive rates of A and D were significantly higher than other capsular serotypes ($P < 0.05$). For strains recovered from the submitted lungs, seven were capsular type A strains, four capsular type D strains and one untypable. For strains from

submitted nasal swabs, one strain was of capsular type A, seven of D, and one strain was untypable.

Identification of *P. multocida* LPS genotypes. LPS genotyping results showed that 124 strains could be of either LPS genotype L3 or L6 (69 and 54 strains respectively), while one strain was untypable (Tab. 1). For isolates recovered from lungs of slaughtered pigs, 40 strains were of LPS genotype L3 and 10 of L6, and 24 strains recovered from slaughtered pig tonsils were of LPS genotype L3 and 29 of L6. For submitted lungs, five strains were of LPS genotype L3 and seven of L6. For isolates from submitted nasal swabs, eight strains belong to LPS genotype L6 and one strain untypable by the LPS-mPCR.

Classification of *P. multocida* strains based on capsular serotype and LPS genotype. Based on capsular type and LPS genotype, 124 strains were classified into six groups, namely A: L3 (capsular type A and LPS genotype L3, 64/124), A: L6 (capsular type A and LPS genotype L6, 16/124), D: L6 (capsular type D and LPS genotype L6, 38/124), F: L3 (capsular type F and LPS genotype L3, 4/124), genotype L3 (untypable capsular type and LPS genotype L3, 1/124), and the type with untypable capsular and LPS (untypable capsular type and untypable LPS genotype, 1/124) (described in Table 1).

Distribution of virulence-associated genes among different genotypes of *P. multocida* strains. On an average, 17 virulence-associated genes were expressed by each strain. The 49 strains observed in this study carrying the highest number of virulence-associated genes (18 virulence-associated genes), were classified into type A: L3 and A: L6, with 33 and 16 strains respectively. The one untypable by both capsular and LPS PCR systems carried the least number (14) of virulence-associated genes. As shown in Table 2, the average number of *P. multocida* virulence-associated genes was 17 or more in addition to capsular type D. The number (1) of unclassified strains was too small to be calculated.

Fourteen virulence-associated genes (adherence proteins: *ptfA*, *fimA*, *hsf-2*; iron-intake related proteins: *exbB*, *exbD*, *tonB*, and *Fur*; Sialic acid kinase: *nanH*; superoxide dismutase: *sodA*, *sodC*; outer membrane proteins: *ompA*, *ompH*, *oma87*, and *plpB*) were de-

Tab. 1. Isolation and identification of *P. multocida* from 801 samples

Sample type	No. of samples examined	Positive rate of samples	No. of strains	No. of unclassified strains	Classification method								
					No. of capsular type			No. of LPS type		No. of capsular type combined LPS type			
					A	D	F	L3	L6	A: L3	A: L6	D: L6	F: L3
Lungs (slaughtered)	421	11.9%	50	0	49	0	1	40	10	39	10	0	1
Tonsil (slaughtered)	180	23.3%	53	0	23	27	3	24	29	21	2	27	3
Lungs (submission)	147	8.2%	12	1 ^a	7	4	0	5	7	4	3	4	0
Nasal swab (submission)	53	17.0%	9	1 ^b	1	7	0	0	8	0	1	7	0
Total	801	15.5%	124	2	80	38	4	69	54	64	16	38	4

Explanations: a – unclassified strains by capsular typing; b – unclassified strains by both capsular and LPS typing

tected in 98% to 100% of all 124 recovered strains. The distribution of *hsf-1*, *pfhA*, *tadD*, *toxA*, *pmHAS*, *hgbA*, *hgbB*, and *nanB* among strains with different genotypes are described in Table 3. *tbpA* was not found in this study. *hsf-1* was found in all D: L6 and F: L3 groups, but was detected in only one strain of capsular type A; *hsf-1* was more frequently found in LPS genotype L6 strains than in L3. *pfhA* was found in 29.8% of the 124 strains, 50% of type A: L3 strains and 100% of type F: L3 were positive for *pfhA*, but it was absent in capsular type D and LPS genotype L6 strains. *tadD* was found in 83.8% of capsular type A strains, with more prevalence in A: L6 strains than A: L3. *tadD* was not found in capsular types D or F, while *toxA* was detected only in two capsular type D strains, accounting for 1.6% (2/124). *pmHAS* was detected in 100% of capsular type A strains, but not in capsular types D or F. *hgbA* was found exclusively in type A: L3 (51.6%). *hgbB* was found in all strains of type D: L6 and F: L3, but only 48.4% in type A: L3. *nanB* was found in all type A: L6 strains and only in another A: L3 (1.6%) type, accounting for 14.5% of the total 124 recovered strains.

P. multocida is a zoonotic pathogen that infects both domesticated and wild animals as well as humans with variable disease outcomes based on host species strain-type. Classification of *P. multocida* strains based on serotyping of capsular and LPS types is time-consuming, serum limited. On the other hand, the PCR typing system established by Townsend for capsular genotype of *P. multocida* strains showed results comparable to the capsular serotyping method, and the LPS-mPCR developed by Harper could classify more strains compared to the LPS serotyping method. In this study, using Townsend and Harpers' strategies, we classified the 124 isolates into six groups based on a combination of capsular typing and LPS genotyping. We believe that our classification method is a more elaborate strategy to facilitate the epidemiological study of *P. multocida*.

As shown in Table 1, the characteristics for capsular type A *P. multocida* strain isolated in the present study

Tab. 2. The average number of virulence-associated genes carried in different capsular types or LPS genotype of *P. multocida* strains

	Type ^a								
	A	D	F	L3	L6	A: L3 ^b	A: L6	D: L6	F: L3
Number of strains	80	38	4	69	54	64	16	38	4
Average number of virulence-associated genes	17.4	16.1	17	17.3	16.6	17.3	18	16.1	17

Explanations: a – capsular types, LPS genotypes; b – capsular type A and LPS genotype 3

Tab. 3. Distribution of eight virulence-associated genes showed differential percentage of expression among different capsular types or LPS genotypes of *P. multocida* strains

Types ^a	Toxic genes							
	<i>hsf-1</i>	<i>pfhA</i>	<i>tadD</i>	<i>toxA</i>	<i>pmHAS</i>	<i>hgbA</i>	<i>hgbB</i>	<i>nanB</i>
Type A (80 stains)	1.3%	40%	83.8%	0%	100%	41.3%	58.8%	21.3%
Type D (38 stains)	100%	0%	0%	5.3%	0%	0%	100%	0%
Type F (4 stains)	100%	100%	0%	0%	0%	0%	100%	0%
Type L3 (69 stains)	7.2%	53.6%	75.4%	0%	94.2%	49.3%	50.7%	1.4%
Type L6 (54 stains)	70.4%	0%	29.6%	3.7%	29.6%	0%	100%	29.6%
Type A: L3 (64 stains)	1.6%	50%	79.7%	0%	100%	51.6%	48.4%	1.6%
Type A: L6 (16 stains)	0%	0%	100%	0%	100%	0%	100%	100%
Type D: L6 (38 stains)	100%	0%	0%	5.3%	0%	0%	100%	0%
Type F: L3 (4 stains)	100%	100%	0%	0%	0%	0%	100%	0%
Total (124 stains)	34.7%	29.8%	55.6%	1.6%	66.1%	27.4%	72.6%	14.5%

Explanations: a – capsular types, LPS genotypes; number of unclassified strains was too small to be calculated separately

was in accordance with that obtained in previous studies, with percent of type A strain higher than that of other types (2, 10, 19). Furthermore, The recovered isolates showed differences in both capsular type and LPS genotype prevalence, based not only on tissue type but the source of the swine donor. For example, capsular type D strain was identified in all tissue-sources of submitted diseased swine tissue but not in slaughtered swine lungs. Generally, slaughtered pigs were healthy adults (> 110 kg body-weight) while submitted swine weighed < 50 kg. Further investigation is necessary to determine whether: 1) adult pigs are resistant to capsular type D strains rendering the strain unable to colonize the adult lung but only tonsils and nasal passages; or, 2) the lungs could only be infected by type D if the resistance declines.

Detection of *P. multocida* virulence-associated genes could provide useful data for pathogenesis study, and some of the virulence related antigens could be candidates for developing effective vaccines against *P. multocida* (1, 3). Virulence-associated genes were highly expressed in *P. multocida* strains analyzed in this study, which agrees well with previous reports (1, 7, 9, 18). However, contrary to previous reports (7, 9), we found *hgbA* to be expressed in 51.6% of *P. multocida* A: L3 strains, and *nanB* in 100% of *P. multocida* A: L6 strains, but no expression in *P. multocida* groups D: L6 and F: L3. The detection rate for *hgbA* and *nanB* in the recovered strains were 27.4% and 14.5%, respectively, lower than that (> 80%) reported by researchers in

Germany (7) and Brazil (9), implying a geographical variation in the two genes in *P. multocida* strains.

toxA, *tbpA* and *pfhA* were regarded as the important epidemiological marker genes for characterizing *P. multocida* field strains (1, 7). *toxA* exists in capsular serotype D strains and is associated with atrophic rhinitis (7, 20). *tbpA*, controlling the receptor for iron binding, which is associated with bovine hemorrhagic septicemia, usually exists in some of the clinical isolates from bovine and sheep (1, 7). *pfhA* encodes the filament like heamoagglutinin, mutation of *pfhA* was reported to decrease the virulence of *P. multocida* to mouse, and inactivation of *fhaB2* resulted in a high degree of attenuation when turkeys were challenged intranasal (8, 23). Among the 124 *P. multocida* isolates, *toxA* was detected exclusively in D: L6 *P. multocida* strains, accounting for 1.6% (2/124). In this study, *pfhA* exists only in *P. multocida* A: L3 and type F: L3 strains, with detection rate 46% and 100%, respectively. Further investigation is necessary to answer whether the virulence of *P. multocida* type A: L3 strain is stronger than A: L6.

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