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Characterization of *Pasteurella multocida* from pneumonic lungs of slaughtered pigs in Korea

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Abstract

Pasteurella multocida is an opportunistic organism that plays a significant role in porcine respiratory disease complex (PRDC). In the current study, we provide nationwide information of *P. multocida* isolates from pneumonic lungs of slaughter pigs by determining their prevalence, subspecies, biovars, capsular types, virulence-associated genes, and minimum inhibitory concentrations. *P. multocida* was the second most frequently confirmed (19.2%) bacterial pathogen and most of the isolates (88.9%) showed simultaneous infection with other respiratory pathogens, especially *Mycoplasma hyopneumoniae* (63.3%, $P<0.001$) and porcine circovirus type 2 (53.3%, $P=0.0205$). Of 42 isolates investigated, 41 (97.6%) were identified as *P. multocida* subspecies *multocida*, and only one isolate was identified as subspecies *septica* (biovar 5). All the isolates were capsular type A and the most prevalent biovar was biovar 3 (40.5%), followed by biovar 2 (31.0%). Comparing virulence-associated genes and biovars, all biovar 2 isolates exhibited $hgbB^{-}pfhA^{+}$ ($P<0.001$); all biovar 3 ($P=0.0002$) and biovar 13 ($P=0.0063$) isolates presented $hgbB^{+}pfhA^{-}$. Additionally, all biovar 2 ($P=0.0037$) isolates and most of biovar 3 ($P=0.0265$) isolates harbored *tadD*. *P. multocida* showed the highest resistance levels to oxytetracycline (73.8%), followed by florfenicol (11.9%). Continuous monitoring is required for surveillance of the antimicrobial resistance and new emerging strains of *P. multocida* in slaughter lines.

Key words : Biovars, Capsular type, Minimum inhibitory concentrations, *Pasteurella multocida* in slaughter pig, Subspecies, Virulence-associated genes

INTRODUCTION

Pasteurella multocida is responsible for a variety of diseases in livestock. Although *P. multocida* is a common inhabitant and an opportunistic organism of the upper respiratory tract in many domestic animals (García et al, 2011; Cardoso-Toset et al, 2013); in pigs, *P. multocida* is a causative agent of progressive atrophic rhinitis (PAR) and plays an important role in porcine respiratory disease complex (PRDC) including porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine circovirus type 2 (PCV2), *Mycoplasma hyopneumoniae* (MHP), and *Mycoplasma hyorhinis* (MHR) (Tang et al, 2009; Hansen et al, 2010; Opriessnig et al, 2011). Respiratory infections in growing-finishing pigs cause huge economic losses and hamper animal welfare. Previous slaughter surveys showed that 20%～25% of finishing pigs suffer from porcine bronchopneumonia (Grest et al, 1997; Maes et al, 2001; Hansen et al, 2010). In three separate studies, the prevalence of *P. multocida* in Korean slaughtered pigs was reported to be 16.4%, 16.9%, and 47.0%, respectively, in pigs with pneumonia. (Kim et al, 1999a; Kim et al, 1999b; Koh et al, 2000).

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P. multocida, a heterogenous organism, is divided into three subspecies (*multocida, septica*, and *gallicida*) and 13 biovars (1～10 and 12～14) determined by carbohydrate fermentation patterns and production of the ornithine decarboxylase (ODC) enzyme (Jamaludin et al, 2005; Varga et al, 2007). Furthermore, five capsular types based on capsular antigens (A, B, D, E, and F) have been reported in *P. multocida*; capsular types A, B, D, and F have been isolated from pigs (García et al, 2011; Cardoso-Toset et al, 2013). The most common strain among swine isolates was the subspecies *multocida*, biovar 3, and capsular types A and D (Blackall et al, 1997; Jamaludin et al, 2005; Tang et al, 2009; García et al, 2011). In Korea, slaughter pig studies have suggested that capsular type A is the most prevalent in porcine pneumonic lungs (Sohn et al, 2009; Sohn et al, 2014). However, there is limited information regarding subspecies and biovars of *P. multocida* isolates in Korean slaughter pigs.

P. multocida has been reported to possess various virulence factors which are associated with pathogenicity such as outer membrane and porin proteins (*oma87, ompH, plpB*, and *psl*), adhesins (*fimA, pfhA, ptfA*, *hsf-1, hsf-2,* and *tadD*), superoxide dismutases (*soda* and *sodC*), iron acquisition-related factors (*exbB, exbBD-tonB, fur, tbpA, hgbA*, and *hgbB*), neuraminidases (*nanB* and *nanH*), hyaluronidases (*pmHAS*), and toxins (*toxA*) (Ewers et al, 2006; Tang et al, 2009; Aski and Tabatabaei, 2016). In addition, it has been reported that there is a correlation between certain virulence factors and capsular types or biovars (Tang et al, 2009; García et al, 2011). Identifying which virulence factors are prevalent is important to understand the pathogenesis of the isolates and to help select potential vaccine candidates.

The increased antimicrobial resistance among disease-causing bacterial pathogens has become a global public health issue and a cause of treatment failure in animal diseases. PRDC is often treated with antimicrobials such as beta-lactams, trimethoprim combination, florfenicol, macrolides, and tetracyclines. However, many studies have reported antimicrobial resistance of *P. multocida* isolates against those antimicrobials (Tang et al, 2009; Lee et al, 2012; Dayao et al, 2014; El Garch et al, 2016). In Korean abattoir studies, *P. multocida* isolates from pigs were susceptible to antimicrobial agents such as ampicillin, ceftiofur, tilmicosin, and enrofloxacin, other than lincomycin and erythromycin (Koh et al, 2000; Sohn et al, 2014).

To our knowledge, only regionally limited studies have been performed to characterize *P. multocida* isolates in Korean slaughter pigs. The objective of this study is to obtain baseline information regarding nationwide *P. multocida* isolates from slaughter pigs by investigating the complexity of respiratory pathogens from pneumonic lungs and determining the distribution and association of capsular types, biovars, extensive virulence-associated gene profiles, and antimicrobial resistance patterns.

MATERIALS AND METHODS

Isolation and identification of porcine respiratory pathogens

A total of 1,039 pneumonic lung samples were obtained from pigs at eight slaughterhouses (233 farms) nationwide for two years (2013～2014). All lung samples were submitted to the Animal and Plant Quarantine Agency for monitoring of respiratory disease pathogens, including *Actinobacillus pleuropneumoniae* (APP), *Bordetella bronchiseptica, Haemophilus parasuis* (HPS), *Streptococcus suis, Trueperella pyogenes*, MHP, MHR, PRRSV, PCV2, and SIV. After morphological and histopathological observation, samples were carried out on 5% sheep blood agar, chocolate agar (Asan Pharm. Co., Ltd., Seoul, Korea), and MacConkey agar (Becton Dickinson, Sparks, USA), then incubated aerobically at 37℃ for 48 h. Presumptive identification of isolates as *P. multocida* was performed by Gram staining and biochemical identification using the VITEK II system (BioMérieux, Marcy l'Etoile, France). The identification was further confirmed by detection of *kmt1* using a species-specific PCR assay (Townsend et al, 1998). All *P. multocida* isolates were maintained at −80℃ until further determination of subspecies, biovars, and capsular types.

Subspecies and biovar determination

P. multocida isolates were subjected to carbohydrate

(sorbitol, dulcitol, maltose, xylose, glucose, trehalose, lactose, and arabinose) fermentation tests and ODC test. Based on sorbitol and dulcitol fermentation, the isolates were classified into one of the three subspecies (*multocida, septica*, and *gallicida*) (Mutters et al, 1985). Furthermore, they were assigned to one of the biovars according to carbohydrate fermentation and ODC activity (Blackall et al, 1997).

PCR assay for capsular typing and virulence-associated gene detection

The isolates of *P. multocida* were cultured overnight in Brain Heart Infusion Broth (BD, Sparks, MD, USA) at 37℃. Genomic DNA was extracted from the growing cells using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Multiplex PCR with capsule-specific primers was performed as previously described to determine the capsular types (A, B, D, E, and F) of the isolates (Townsend et al, 2001). PCR analysis of 22 virulence-associated genes (*oma87, ompH, plpB, psl, fimA, pfhA, ptfA, hsf-1, hsf-2, tadD, sodA, sodC, exbB, exbBD-tonB, fur, tbpA, hgbA, hgbB, nanB, nanH, pmHAS*, and *toxA*) were investigated according to previous studies (Ewers et al, 2006; Atashpaz et al, 2009; Tang et al, 2009; Aski and Tabatabaei, 2016). PCR was performed using a Mastercycler ep Gradient S (Eppendorf, Hamburg, Germany) and the PCR-generated products were analyzed with a capillary electrophoresis system (QIAxcel Advanced System; Qiagen). PCR assays were repeated in parallel with the relevant positive and negative controls.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) values for *P. multocida* isolates (n=42) were determined by the broth microdilution method using commercially prepared 96-well antimicrobial testing plates containing 18 different agents (BOPO6F; TREK Diagnostic System, West Sussex, UK) according to the manufacturer's instructions for *P. multocida*. The following antimicrobials were tested: penicillin, ampicillin, ceftiofur, florfenicol, gentamicin, neomycin, chlortetracycline, oxytetracycline, clindamycin, enrofloxacin, danofloxacin, trimethoprim/sulfamethoxazole, sulfadimethoxine, spectinomycin, tulathromycin, tylosin tartrate, tilmicosin, and tiamulin. *Escherichia coli* ATCC 25922 was also included as a quality control. MICs were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for oxytetracycline, florfenicol, penicillin, ampicillin, enrofloxacin, tulathromycin, ceftiofur, and tilmicosin, or a previous study for trimethoprim/sulfamethoxazole, for which CLSI breakpoints were not available (CLSI, 2015; El Garch et al, 2016). Overall MIC_{50} and MIC_{90} values (i.e., the lowest concentrations at which growth was inhibited by 50 and 90%, respectively) of each antimicrobial for all isolates were determined.

Table 1. Distribution of detected respiratory pathogens and the prevalence of *Pasteurella multocida* in pneumonic lung sample at abattoir in Korea from 2013 to 2014

	No. of sample analyzed	Bacteria[a]								Virus[b]			None[c]
		PM	MHP	MHR	APP	SS	TP	BB	HPS	PCV2	PRRSV	SIV	
Total no. (%) of sample from which respiratory pathogens were determined	1,039	199 (19.2)	434 (41.8)	176 (16.9)	51 (4.9)	41 (3.9)	5 (0.5)	2 (0.2)	1 (0.1)	477 (45.9)	222 (21.4)	44 (4.2)	218 (21.0)
Total no. (%) of sample co-infected with *P. multocida*	199	199 (100)	126 (63.3)	49 (24.6)	5 (2.5)	12 (6.0)	0 (0)	0 (0)	0 (0)	106 (53.3)	55 (27.6)	11 (5.5)	

[a]PM, *Pasteurella multocida* MHP, *Mycoplasma hyopneumoniae* MHR, *Mycoplasma hyorhinis* APP, *Actinobacillus pleuropneumoniae* SS, *Streptococcus suis* TP, *Trueperella pyogenes* BB, *Bordetella bronchiseptica* HPS, *Haemophilus parasuis*.
[b]PCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus; SIV, swine influenza virus.
[c]None, none of the respiratory bacteria and virus was detected in this study.

Statistical analysis

Statistical testing was performed with GraphPad Prism, version 5.01 (MDF Co. Ltd., Tokyo, Japan). The Pearson chi-squared and Fisher's exact tests were used to assess the association among biovars and virulence-associated genes. P value of <0.05 was considered as significant.

RESULTS

Prevalence of *P. multocida* in pneumonic lungs of slaughter pigs

In the 1,039 pneumonic lung samples of respiratory pathogens monitored, *P. multocida* was isolated from 19.2% (n=199) (Table 1), being the second most frequently detected bacterial pathogen in this study. Most of the samples (88.9%, 177/199) showed coinfections with other respiratory pathogens such as MHP (63.3%), PCV2 (53.3%), PRRSV (27.6%), or MHR (24.6%). S. suis, SIV, and APP were identified to a lesser extent, 6.0%, 5.5%, and 2.5%, respectively. Among the 199 *P. multocida* isolates, 42 were randomly selected and characterized in this study.

Table 2. Distribution of capsular type, subspecies, and biovars of *Pasteurella multocida* (n=42)

Capsular type	Subspecies	Biovar
Capsular type A (n = 42)	*Multocida* (97.6%, n=41)	Biovar 2 (31.0%, n=13)
		Biovar 3 (40.5%, n=17)
		Biovar 13 (26.2%, n=11)
	Septica (2.4%, n=1)	Biovar 5 (2.4%, n=1)

Capsular types, subspecies, and biovars

The results of the detection of subspecies, biovars, and capsular types among the studied isolates are presented in Table 2. All 42 isolates belonged to capsular type A. Most of them (97.6%, n=41) were identified as *P. multocida* subspecies *multocida*, which produces acid from sorbitol and glucose, but not from dulcitol, lactose, and maltose. Interestingly, only one isolate was determined to be of subspecies septica (biovar 5) which is ODC-positive and can produce acid from glucose and xylose, but not from arabinose, dulcitol, sorbitol, trehalose, lactose, and maltose. Among the ODC-positive isolates (n=31), more than half (54.8%, n=17) belonged to biovar 3, followed by biovar 2 (n=13), and biovar 5 (n=1). ODC-negative strains (n=11) were identified as biovar 13.

Distribution of virulence-associated genes

According to the 22 virulence-associated gene analysis, all *P. multocida* isolates harbored 16 genes *(oma87, psl, ompH, sodA, sodC, ExbB-tonB, hgbA, nanB, nanH, hsf-2, plpB, fur, fimA, exbB, ptfA*, and *pmHAS*). None of the isolates were positive for *tbpA, toxA, and hsf-1*. The prevalence of *tadD* (69.0%), *hgbB* (66.7%), and *pfhA* (33.3%) was variable. The distribution of virulence-associated genes according to the biovars is shown in Table 3. All the isolates of biovar 2 possessed *pfhA* ($P<0.001$) and *tadD* ($P<0.01$). All the isolates of biovars 3 ($P<0.001$) and 13 ($P<0.01$) had *hgbB*. Contrary to biovar 13, most biovar 3 isolates (88.2%, n=15) harbored *tadD*.

Table 3. Distribution of biovars within the following virulence-associated (VA) genes

Biovars	No. (%) of VA genes within the following biovars		
	tadD (n = 29, 69.0%)	*hgbB* (n = 28, 66.7%)	*pfhA* (n = 14, 33.3%)
Biovar 2 (n=13)	13 (44.8)**	0	13 (92.9)***
Biovar 3 (n=17)	15 (51.7)*	17 (60.7)***	0
Biovar 5 (n=1)	1 (3.4)	0	1 (7.1)
Biovar 13 (n=11)	0	11 (39.3)**	0

*$P<0.05$, **$P<0.01$, ***$P<0.001$.

[a]All isolates possessed the following genes: *oma87, psl, ompH, sodA, sodC, ExbB-tonB, hgbA, nanB, nanH, hsf-2, plpB, fur, fimA, exbB, ptfA*, and *pmHAS*. However, *tbpA, toxA*, and *hsf-1* were not found in any of the isolates.

Antimicrobial susceptibility

The cumulative MIC distribution of the 42 *P. multocida* isolates from slaughter pigs, the resistant rates in each antimicrobial as well as the MIC_{50} and MIC_{90} are summarized in Table 4. Among the 18 antimicrobials tested, the most prevalent isolates determined were resistant to oxytetracycline (73.8%), followed by florfenicol (11.9%), penicillin (4.8%), ampicillin (4.8%), and enrofloxacin (2.4%). None of the isolates showed resistance to trimethoprim/sulfamethoxazole, tulathromycin, ceftiofur, and tilmicosin.

DISCUSSION

P. multocida was the second most frequently detected bacterial pathogen (19.2%) from pneumonic lungs of slaughter pigs in this study. This result was similar to that of two previous studies conducted in Korea, which reported an isolation rate of 16.4% and 16.9%, respectively (Kim et al, 1999a; Koh et al, 2000). However, this is relatively higher than the isolation rate (6.4%) reported in a New Zealand survey (Jamaludin et al, 2005). Most of *P. multocida* isolates (88.9%) showed coinfection with other respiratory pathogens, particularly MHP (63.3%, $P<0.001$) and PCV2 (53.3%, $P=0.0205$). Therefore, veterinarians should consider that coinfection with other respiratory pathogens may exist in each herd to control PRDC in finishing pigs.

In this study, 42 isolates were selected randomly to investigate their subspecies, biovar, capsular type, virulence-associated genes, and MIC. This is the first subspecies and biovar survey in Korean slaughter pigs. Almost all *P. multocida* isolates (n=41, 97.6%) were of subspecies *multocida*. Biovar 3 (n=17, 40.5%) and 2 (n=13, 31.0%) were the most common in this study, which showed a predominance similar to that reported in previous studies (Jamaludin et al, 2005; Varga et al,

Table 4. Antimicrobial susceptibility and cumulative percentage of *Pasteurella multocida* isolates (n=42) inhibited by 18 antimicrobials

Antimicrobial	Cumulative percentage of strains inhibited at antimicrobial concentration (μg/mL) of:													MIC_{50}[a] (μg/mL)	MIC_{90}[a] (μg/mL)	S (%)[b]	I (%)[b]	R (%)[b]	Reference
	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	≥512						
Oxytetracycline			7.1	26.2	81.0			100						2	16	7.1	19	73.8	CLSI
Florfenicol		21.4	83.3	85.7		88.1	100							0.5	8	85.7	2.4	11.9	CLSI
Penicillin	81.0	95.2					100							≤0.12	0.25	95.2	0	4.8	CLSI
Ampicillin		95.2					97.6		100					≤0.25	≤0.25	97.6	0	4.8	CLSI
Enrofloxacin	97.6			100										≤0.12	≤0.12	97.6	0	2.4	CLSI
Trimethoprim/sulfamethoxazole					100									≤2	≤2	NDc	ND	0	El Garch et al. 2016
Tulathromycin				97.6	100									≤1	≤1	100	0	0	CLSI
Ceftiofur		100												≤0.25	≤0.25	100	0	0	CLSI
Tilmicosin						97.6	100							≤4	≤4	100	0	0	CLSI
Chlortetracycline			7.1	38.1	92.9	100								2	2	ND	ND	ND	-
Spectinomycin							21.4	97.6			100			16	16	ND	ND	ND	-
Clindamycin					2.4		14.3	100						16	16	ND	ND	ND	-
Danofloxacin	97.6			100										≤0.12	≤0.12	ND	ND	ND	-
Gentamicin				14.3	78.6	95.3		100						2	4	ND	ND	ND	-
Neomycin						71.4	92.9			100				≤4	8	ND	ND	ND	-
Sulfadimethoxine												61.9	100	≤256	≥512	ND	ND	ND	-
Tiamulin				2.4		4.8		57.2	100					16	32	ND	ND	ND	-
Tylosin tartrate				2.4		4.8	23.8	80.9	100					16	32	ND	ND	ND	-

The gray color indicated the concentration range of each antimicrobial in the BOPO6F plate. And susceptible and resistance breakpoints are indicated in double vertical and single vertical lines, respectively.

[a]MIC50 and MIC90 concentrations at which the growth of 50% and 90%, respectively, of the isolates is inhibited.

[b]S, susceptible; I, intermediate; R, Resistant.

[c]ND, Not determined.

2007; García et al, 2011). The prevalence of biovar 13 was 26.2% (n=11) in the present study, which is much higher than previous studies (2.0%～4.8%) (Blackall et al, 1997; Varga et al, 2007). Only one isolate belonged to subspecies septica also identified as biovar 5. *P. multocida* subspecies *septica*, related with dog bites and cat claws, has been reported to have low prevalence (Jamaludin et al, 2005; Varga et al, 2007). All isolates were capsular type A, which is associated with the predominance of capsular type A in pneumonic pig lungs (Choi et al, 2001; García et al, 2011; Lee et al, 2012).

Virulence-associated genes are important for understanding the pathogenesis of *P. multocida*, and the wide distribution of these genes is suggested to be related with the survival and infection of *P. multocida* within the host environment (Tang et al, 2009; Aski and Tabatabaei, 2016). In agreement with previous studies, all the isolates possessed *oma87, psl, ompH, sodA, sodC, ExbB-tonB, hgbA, nanB, nanH, hsf-2, plpB, fur, fimA, exbB,* and *ptfA*, while none possessed *tbpA* (Ewers et al, 2006; Tang et al, 2009; García et al, 2011). On the other hand, previous studies have reported that some virulence-associated genes are significantly related to specific capsular types; *toxA* and *hsf-1* are often associated with capsular type D, and *pmHAS* is associated with type A (Ewers et al, 2006; Tang et al, 2009). In agreement with those results, all isolates in this study exhibited virulence-associated gene profile of $pmHAS^{+}$ $toxA^{-}hsf\text{-}1^{-}$. Similar to a previous study, distinctive correlations were detected between the *hgbB/pfhA* and biovar 2/3 (García et al, 2011). In this study, all biovar 2 isolates showed $hgbB^{-}pfhA^{+}$ ($P<0.001$), and all biovar 3 ($P=0.0002$) and biovar 13 ($P=0.0063$) isolates exhibited $hgbB^{+}pfhA^{-}$. In addition, all biovar 2 ($P=0.0037$) isolates and most of biovar 3 ($P=0.0265$) isolates presented *tadD*. However, there is no previous report to compare biovars and *tadD*. To understand the associations between biovar 2/3 and *tadD*, further studies with various *P. multocida* isolates are required.

Antimicrobial therapy, including beta-lactams (ampicillin, penicillin, and cephalosporins); trimethoprim combination; florfenicol; macrolides (erythromycin, tilmicosin, and tulathromycin); and tetracyclines is the best treatment for PRDC (Dayao et al, 2014). In the current study, *P. multocida* showed high susceptibility (>90%) to beta-lactams (ampicillin, penicillin, and ceftiofur); macrolides (tilmicosin and tulathromycin); and fluoroquinolone (enrofloxacin). Therefore, these antimicrobials are recommended for the treatment of *P. multocida* infection at growing-finishing pigs in case of the absence of antimicrobial susceptibility test. Many studies have reported tetracycline is the most resistant antimicrobial (Portis et al, 2013; Dayao et al, 2014; Sweeney et al, 2017). Our study is in concordance with those results, showing high resistance to tetracycline (73.8%), relatively higher than the rates reported in China (58.0%) and North America (60.0%) (Tang et al, 2009; Sweeney et al, 2017), and much higher than the rates reported in Australia (28.0%), EU (20.4%) and Czech Republic (32.2%) (Dayao et al., 2014; El Garch et al., 2016; Nedbalcová and Kučerová, 2013). Florfenicol has been suggested as a treatment for *P. multoicda* infection, due to low resistance rates (0%～2.0%) in Australia, China, Czech Republic, EU, and North America (Tang et al, 2009; Nedbalcová and Kučerová, 2013; Dayao et al, 2014; El Garch et al, 2016; Sweeney et al, 2017). Contrary to the previous studies, the resistance rate of florfenicol (11.9%) was relatively higher in our study. According to the Korea Animal Health Products Association, tetracyclines and florfenicol are the most consumed antimicrobials in the Korean pig industry (APQA, 2016). Therefore, those antimicrobials should be used carefully because their high resistance rates are probably due to frequent use. Additionally, continuous surveillance of antimicrobial resistance is needed for selection of appropriate agents and the emergence of new resistant isolates.

This is the first extensive study of *P. multocida* isolates from lungs of slaughter pigs in Korea. The current findings will help to understand *P. multocida* as one of the PRDC agents in finishing pigs and provide scientific information of *P. multocida* affecting Korean slaughter pigs.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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