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Genetic and phenotypic characterization of tetracycline-resistant *Pasteurella multocida* isolated from pigs

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ABSTRACT

Pasteurella multocida causes single or complex respiratory disease in pigs. Although antimicrobial therapy is the most effective treatment for porcine respiratory disease, *P. multocida* shows increased antimicrobial resistance in Korea. Therefore, we aimed to investigate the phenotypic and genotypic characterization of tetracycline-resistant *P. multocida*. Thirty-seven of 454 *P. multocida* isolates from South Korea between 2010 and 2016 were selected. Four *tet* genes [*tet*(B) (78.4%), *tet*(H) (16.2%), *tet*(C) (5.4%), and *tet*(O) (2.7%)] were observed. This is the first report of *tet*(C) in *P. multocida*. Various virulence factors were observed in both tetracycline-resistant and -susceptible *P. multocida* isolates. Genes encoding *pmHAS* and *pfhA* were more prevalent in tetracycline-resistant than in tetracycline-susceptible isolates. Some virulence factors exhibited association with serogroups. *tadD* and *sodA* were common in serogroup A, while *hsf-l* was significantly associated with serogroup D ($p < 0.01$). Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) results showed the genetic diversity of tetracycline-resistant *P. multocida*. MLST showed six different sequence types (ST), with clonal complex 13 encompassing 56.8% of the strains. PFGE was more efficient in differentiating the isolates, and 29 PFGE patterns of the strains were observed. By combining these methods, identical STs and PFGE patterns were observed in isolates from different farms, suggesting that transmission of antimicrobial-resistant *P. multocida* strains between farms might occur in a geographically discrete population. In future, epidemiological approaches and development of effective vaccines should focus on the major clonal lineages carrying the important virulence factors and frequently observed resistance genes to prevent the transmission and control the disease.

1. Introduction

Pasteurella multocida is the most commonly identified bacterial pathogen of porcine respiratory disease. *P. multocida* strains are grouped into five serogroups (A, B, C, D, and E) based on lipopolysaccharide antigens (Townsend et al., 2001). Non-toxigenic type A strains and a few type D and/or toxigenic strains may play a crucial role in porcine respiratory disease complex, which is regarded as one of the most common and costly diseases of intensively housed pigs. The virulence factors of *P. multocida* that have been identified include diverse adhesins, toxins, iron acquisition proteins, neuraminidases, and outer membrane proteins (Aski and Tabatabaei, 2016).

It is widely accepted that specific serotypes and pathotypes of *P. multocida* strains are responsible for most respiratory disease syndromes in pigs that are associated with pneumoniae, atrophic rhinitis, and/or mycoplasma infection (Davies et al., 2003). However, the distribution and prevalence of pathotypes and genotypes can vary considerably between regions and over time in a particular region. Different methods have been used in studies analyzing the genetic characteristics of *P. multocida*. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are considered appropriate techniques in terms of discriminatory power and reproducibility for genetic typing of microorganisms.

Antimicrobial therapy is still the most effective tool for the treatment of pasteurellosis; however, recently there has been an increase in resistance to antimicrobial drugs, especially tetracycline and sulfonamides that are commonly used in the pig industry. Owing to the widespread use of tetracycline, most of the bacterial species causing diseases in animals have developed resistance to tetracycline. To date, 73 tetracycline resistance genes are known (https://faculty.washington.edu/marilynr/). Among them, *tet*(B), *tet*(H), *tet*(G), and *tet*(L) have been detected in *P. multocida* of veterinary origin (Michael et al., 2018).

In Korea, *P. multocida* is one of the most common pathogens causing single or complex respiratory disease in pigs (Lee et al., 2012; Cheong

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et al., 2017). The antimicrobial resistance rate of *P. multocida* isolated from Korea is higher than that of *P. multocida* isolated from other countries (Oh et al., 2018). Data on pathotypes and genotypes of resistant *P. multocida* from diseased pigs may provide a better understanding of pasteurellosis. However, little information is available on the antimicrobial resistance of *P. multocida*. Therefore, in this study, we aimed to investigate the distribution of virulence factors, resistance genes, and genetic relatedness of tetracycline-resistant *P. multocida* from pigs over a period of 7 years.

2. Materials and methods

2.1. Bacterial strains

A total of 454 *P. multocida* isolates were collected from all provinces in Korea from 2010 to 2016: 87 from the Animal and Plant Quarantine Agency and 367 from nine laboratories/centers participating in the Korean Veterinary Antimicrobial Resistance Monitoring System. These *P. multocida* strains were isolated from nasal swabs and lungs of diseased pigs from 282 farms throughout Korea in 2010 (n = 36), 2011 (n = 51), 2012 (n = 45), 2013 (n = 32), 2014 (n = 39), 2015 (n = 60), and 2016 (n = 19). For each farm, one to five isolates were retained for antimicrobial susceptibility testing. The bacteria were isolated on Columbia agar (Becton Dickinson, Sparks, MD) with 5% sheep blood, and suspected colonies were identified by polymerase chain reaction (PCR) (Townsend et al., 2001) or Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF; bioMerieux, Marcy L'Etoile, France). A multiplex capsular PCR based on capsule-specific primers (CAPA, CAPB, CAPD, CAPE, and CAPF) was used as described by Townsend et al. (2001).

Minimum inhibitory concentration (MIC) values for all isolates were determined using a broth microdilution method with 96-well microtiter plates (BOPO6, Sensititre, Trek diagnostic Systems Inc., East Grinstead, UK) according to the Clinical and Laboratory Standards Institute (CLSI, 2015) standards. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. The resistance to tetracycline was interpreted on the basis of breakpoints provided by the CLSI (2013). In total, 37 isolates among 167 tetracycline-resistant isolates and 21 isolates among 287 tetracycline-susceptible isolates were selected to represent only one isolate per farm, in order to obtain a collection of epidemiologically unrelated strains.

2.2. Detection of tetracycline resistance genes and virulence genes

The DNA templates were prepared from an overnight culture of *P. multocida* by boiling methods as described previously (Dayao et al., 2016). The *tet* genes [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(H), *tet*(L), *tet* (M), and *tet*(O)] were detected in the genomic DNA using PCR as described previously (Dayao et al., 2016). The nucleotide sequences of *tet* (C) were compared with those of the corresponding gene of plasmid pSE11-1 from *Escherichia coli* pSE11 (NG_048181). Detection of 22 virulence-associated genes, including adhesion-related genes (*ptfA, fimA, hsf-1, hsf-2, pfhA, tadD*), genes for dermonecrotic toxin (*tox*A), iron acquisition (*exbB, exbD-tonB, fur, hgbA, hgbB, tbpA*), neuraminidases (*nanB, nanH*), hyaluronidase (*pmHAS*), outer membrane proteins (*ompH, ompA, Oma87, plpB*), and superoxide dismutases (*sod*A, *sod*C), was performed as described previously (Aski and Tabatabaei, 2016).

2.3. Conjugation experiment

The transferability of *tet* genes was assessed by solid agar mating using sodium azide-resistant *E. coli* J53 as described previously, with slight modifications (San Millan et al., 2009). Briefly, overnight cultures of 37 tetracycline-resistant donor isolates and *E. coli* J53 recipient strain were inoculated with fresh tryptic soy broth (Becton Dickinson) and cultured for 4 h. The freshly cultured bacteria were mated with a donor/recipient ratio of 1:4. Drops of these mixtures were placed on a tryptic soy agar (Becton Dickinson) plate containing no antibiotics and incubated overnight at 37°C. After incubation, the mating mixtures were spread on tryptic soy agar plates containing tetracycline (2 mg/L) and sodium azide (150 mg/L).

2.4. Molecular typing of tetracycline-resistant isolates

Genetic analysis of isolates carrying *tet* genes was performed by PFGE and MLST. PFGE was carried out as described previously (Pors et al., 2011). Briefly, genomic DNA was prepared in agarose blocks, digested with *Apa*I enzyme (Takara Bio Inc, Shiga, Japan), and resolved in the CHEF Mapper apparatus (Bio-Rad laboratories, Hercules, CA). The results were analyzed using BioNumerics software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). MLST based on seven housekeeping genes *adk, est, pmi, zwf, mdh, gdh,* and *pgi* was performed according to the method described by Subaaharan et al. (2010). Sequencing of PCR products was performed at Macrogen Inc. (South Korea) using PCR primers. MLST alleles and the resulting sequence types (STs) were assigned by submission of the respective data to multi-host *P. multocida* MLST data base (http://pubmlst.org/pmultocida/). The new sequences were submitted to the *P. multocida* MLST data base and assigned as new MLST.

2.5. Statistical analysis

The distribution of virulence-associated genes among capsular serogroups was compared by Fisher's exact test or the x^2 test. A value of $P < 0.01$ was considered significant.

3. Results

The genetic basis of the observed tetracycline resistance phenotypes was tested by PCR for nine *tet* genes. As shown in Table 1, the tetracycline resistance genes *tet*(B) (78.4%), *tet*(H) (16.2%), *tet*(C) (5.4%), and *tet*(O) (2.7%) were detected whereas none of the isolates harbored any of the tetracycline resistance genes *tet*(A), *tet*(D), *tet*(E), *tet*(L), or *tet* (M). All tetracycline-resistant isolates, except for two isolates, were also resistant to 1–3 other antimicrobial agents (Table 1). None of the tetracycline resistance genes were transferred to the *E. coli* J53 recipient *via* conjugation.

Twenty-two virulence genes were highly prevalent in both tetracycline-resistant and -susceptible isolates, except *toxA* (Table 2). However, some genes were prevalent in tetracycline-resistant isolates or specific serotype groups. The prevalence of *pmHAS* (100% *vs.* 14.3%) and *pfhA* (59.5% *vs.* 23.8%) was significantly different in the tetracycline-resistant and -susceptible isolates, respectively ($p < 0.01$). Some of the virulence factors exhibited association with serogroups; *tadD* and *sodA* were less common in serogroup D than in serogroup A ($p < 0.01$). In contrast, *hsf-l* was significantly associated with serogroup D ($p < 0.01$).

A total of six different STs were identified in 37 tetracycline-resistant isolates. The largest group was clonal complex 13, and it comprised ST13 (n = 13) and ST44 (n = 8). New ST (41-53-35-64-4-3-13, assigned as ST347) was detected in five isolates. The predominant STs differed with time. ST286 (36.4%, 4/11) and ST44 (36.8%, 7/19) were more frequently observed in the early stage (2010–2012) and late stage (2015–2016), respectively. Capsular types and STs were strongly related; all capsular type A isolates belonged to five STs (ST13, ST44, ST74, ST286, and ST347) and five capsular type D isolates belonged to one ST (ST50). PFGE patterns showed more genetic diversity than MLST. Twenty-nine PFGE patterns (A—AC) were observed in 37 tetracycline-resistant isolates. By combining both methods, identical ST and PFGE patterns (ST286-AB, ST13-H, and ST44-J) were observed in isolates from different farms located in different provinces.

Table 1

Characterization of tetracycline resistant *P. multocida* isolated from pigs.

Isolates	Farm ID	Province	Isolation Year	MIC (μg/ml)		Tetracycline resistance genes	Capsular type	Additional antimicrobial resistance [a]	No. of carried virulence factors	ST type[a]	PFGE[a]
				CTC	OTC						
V01-10-070	a	Gyeonggi	2010	4	16	*tet*(B)	A	SDM	17	286	AC
V01-12-026	b	Jeonnam	2011	8	16	*tet*(B), *tet*(H)	A	SDM, FFC	17	347	W
V06-11-052	c	Jeonbuk	2011	2	16	*tet*(B)	A	SDM	17	13	K
V06-11-055	d	Jeonbuk	2011	1	16	*tet*(C)	A	–	18	13	S
V02-12-005	e	Gyeonggi	2012	4	16	*tet*(B)	A	PEN, SDM	17	286	AB
V02-12-007	f	Gyeonggi	2012	2	16	*tet*(B)	A	AMP, PEN, SDM, SPT	17	44	L
V06-12-007	g	Jeonbuk	2012	2	16	*tet*(C)	D	SDM	18	50	V
V06-12-031	h	Jeonbuk	2012	4	16	*tet*(B)	A	SDM, FFC	16	286	AB
V06-12-048	i	Jeonbuk	2012	4	16	*tet*(B)	A	PEN, SDM	17	286	AB
V06-12-052	j	Jeonnam	2012	2	16	*tet*(B)	A	SDM	17	13	O
V07-12-003	k	Jeonnam	2012	4	16	*tet*(B)	D	SDM, SXT, SPT	18	50	T
V06-13-001	l	Chungnam	2013	2	16	*tet*(B)	A	SDM	19	13	P
V01-14-043	m	Jeonbuk	2014	2	16	*tet*(B)	A	SDM	18	13	I
V01-14-045	n	Jeonbuk	2014	2	16	*tet*(B)	A	SDM, FFC	18	13	H
V01-14-046	o	Jeonbuk	2014	2	16	*tet*(B)	A	SDM, FFC	16	13	H
V01-14-047	p	Jeonnam	2014	2	16	*tet*(B)	A	FFC	18	13	N
V01-14-024	q	Chungnam	2014	2	16	*tet*(B)	A	SDM	18	13	H
V01-14-026	r	Chungbuk	2014	4	16	*tet*(B)	D	SDM	18	50	U
V01-15-027	s	Gangwon	2015	4	16	*tet*(B)	D	SDM	18	50	E
V01-15-034	t	Gyeonggi	2015	4	16	*tet*(B)	A	PEN, SDM	16	286	Z
V01-15-036	u	Gyeongbuk	2015	2	16	*tet*(B)	A	SDM	18	13	M
V01-15-037	v	Jeju	2015	4	16	*tet*(H)	A	SDM, FFC	17	347	F
V01-15-039	w	Gyeongbuk	2015	1	16	*tet*(H)	A	SDM	18	44	C
V01-15-041	x	Gyeonggi	2015	2	16	*tet*(B)	D	SDM, SXT, FFC	17	50	D
V01-15-051	y	Jeonbuk	2015	2	16	*tet*(B)	A	SDM, FFC	18	13	Y
V01-15-053	z	Chungnam	2015	4	16	*tet*(H)	A	SDM, FFC	17	347	H
V04-15-022	aa	Gyeonggi	2015	2	16	*tet*(B)	A	SDM	17	13	G
V04-15-038	ab	Chungnam	2015	2	16	*tet*(B)	A	AMP, PEN, SDM, SPT	18	13	H
V04-15-044	ac	Chungbuk	2015	16	16	*tet*(O)	A	TIL, TUL, PEN SDM, FFC	17	74	Q
V06-15-008	ad	Jeonbuk	2015	2	16	*tet*(B)	A	SDM	18	44	AA
V06-15-011	ae	Jeonbuk	2015	2	16	*tet*(B)	A	SDM	18	44	J
V06-15-013	af	Jeonbuk	2015	2	16	*tet*(B)	A	SDM	18	44	J
V03-16-002	ag	Gangwon	2016	4	16	*tet*(B)	A	SDM, FFC	18	44	J
V06-16-003	ah	Jeonbuk	2016	4	16	*tet*(H)	A	SDM, FFC	18	347	A
V06-16-023	ai	Jeonbuk	2016	2	16	*tet*(B)	A	AMP, PEN, SPT	19	44	R
V10-16-005	aj	Jeju	2016	4	16	*tet* (H)	A	SDM, FFC	18	347	X
V10-16-007	ak	Jeju	2016	2	16	*tet*(B)	A	–	18	44	B

[a] Abbreviations: CTC, chlortetracycline; OTC, oxytetracycline; SDM, **S**ulphadimethoxine; FFC, florfenicol; PEN, penicillin; AMP, ampicillin; SPT, spectinomycin; SXT, trimethoprim/sulfamethoxazole; TIL, tilmicosin; TUL, tulathromycin; ST, sequence type; PFGE, pulsed-field gel electrophoresis.

4. Discussion

In this study, we aimed to investigate the phenotypic and genotypic characterization of tetracycline-resistant *P. multocida* and found that virulence factors were carried by tetracycline-resistant and -susceptible *P. multocida* isolates. Different pathotypes and genotypes of tetracycline-resistant *P. multocida* are prevalent in pig farms. Four different *tet* genes were detected in 37 tetracycline-resistant *P. multocida* isolates. The presence of *tet*(B), *tet*(H), and *tet*(C) suggested that the mechanism underlying the resistance to tetracycline involves efflux pump proteins that expel drugs out of the cell, leading to inactivity of tetracycline against the bacterial pathogens. The gene *tet*(B) was the most frequently found *tet* gene (78.4%). This finding is consistent with that of a previous study, wherein *tet*(B) (85.7%) was reported to be the predominant gene in isolates from Australia in 2010 (Dayao et al., 2016). Furthermore, *tet* (B) is the most common efflux gene of gram-negative bacteria (Roberts, 2005). Since 1990, tetracycline resistance has been most frequently found in Korean isolates of *P. multocida* (Choi et al., 2001; Lee et al., 2012). Thus, the increase in tetracycline resistance in Korea may be associated with the dissemination of *tet*(B) among *P. multocida* isolates.

The *tet*(C) gene was identified for the first time in *P. multocida* species in this study. This gene showed 100% match with *E. coli* SE11 pSE11-1 *tet*(C) gene encoding tetracycline efflux MFS transporter. In pigs, *tet*(C) gene has been reported in tetracycline-resistant *Chlamydia suis* strains, which are found in the intestinal tract (Dugan et al., 2004). Further studies on the function and structure of this gene in *P. multocida* strains are needed for elucidation of its association with tetracycline resistance.

Many tetracycline resistance genes have been found in transmissible plasmids and transposons (Roberts, 2005; Michael et al., 2018). Especially, the major genes found in this study, *tet*(B) and *tet*(H), were carried on Tn*10* on pB1001 and Tn*5706* on pPMT1, respectively (Michael et al., 2018). These mobile elements may have played a major role in the spread of tetracycline resistance. Although none of the tetracycline resistance genes were transferred *via* conjugation in this study, further studies on the elements carrying the *tet* genes are needed to understand the spread of tetracycline resistance.

Overall, we identified several virulence factors in both tetracycline-resistant and -susceptible isolates. However, the genes for *pmHAS* and *pfhA* were more frequently found in tetracycline-resistant and -susceptible isolates, respectively. Further studies are needed to investigate the association between these virulence factors and tetracycline resistance. Most of the virulence genes were equally distributed in serogroups A and D; however, a few virulence factors exhibited a distinctive association with serogroups. *tadD* and *sodA* were less common in serogroup D than in serogroup A. This result is consistent with that of a previous study by Katsuda et al. (2013), who reported the presence of *tadD* gene in 94.1% of capsular type A strains and only in 12.5% of type

Table 2
Prevalence of virulence factors of *P. multocida* isolated from pigs.

Virulence factors	No. (%) of virulence factors within the following tetracycline-resistant and -susceptible isolates			
	Tetracycline-resistant isolates (n = 37)		Tetracycline-susceptible isolates (n = 21)	
	Capsular A (n = 32)	Capsular D (n = 5)	Capsular A (n = 18)	Capsular D (n = 3)
Adhesins				
ptfA	32 (100)	5 (100)	18 (100)	3 (100)
fimA	32 (100)	5 (100)	18 (100)	3 (100)
hsf-1	0 (0)	5 (100)	1 (5.6)	3 (100)
hsf-2	32 (100)	5 (100)	18 (100)	3 (100)
pfhA	22 (68.8)	0 (0)	3 (16.7)	2 (66.7)
tadD	27 (84.4)	0 (0)	15 (83.3)	0 (0)
Demonecrotic toxins				
toxA	1 (3.1)	0 (0)	1 (5.6)	0 (0)
Iron acquisition				
exbB	32 (100)	5 (100)	18 (100)	3 (100)
exbD-tonB	32 (100)	5 (100)	18 (100)	3 (100)
fur	32 (100)	5 (100)	18 (100)	3 (100)
hgbA	32 (100)	5 (100)	18 (100)	3 (100)
hgbB	11 (34.4)	4 (80.0)	14 (77.8)	3 (100)
tbpA	24 (75.0)	5 (100)	15 (83.3)	2 (66.7)
Neuraminidase				
nanB	32 (100)	5 (100)	18 (100)	3 (100)
nanH	32 (100)	5 (100)	18 (100)	3 (100)
Hyaluronidase				
pmHAS	32 (100)	5 (100)	3 (16.7)	0 (0)
Outer membrane proteins				
ompH	32 (100)	5 (100)	18 (100)	3 (100)
ompA	32 (100)	5 (100)	18 (100)	3 (100)
Oma87	30 (93.8)	5 (100)	13 (72.2)	2 (66.7)
plpB	32 (100)	5 (100)	18 (100)	3 (100)
Superoxide dismutases				
sodA	23 (71.9)	0 (0)	15 (83.3)	1 (33.3)
sodC	26 (81.3)	5 (100)	17 (94.4)	3 (100)

D strains. However, *sodA* gene was present in 100% of the strains of both capsular type A and type D (Katsuda et al., 2013; Aski and Tabatabaei, 2016). Although only five isolates belonging to the serogroup D were detected in this study, *hsf-l* was significantly associated with serogroup D. This result was similar to that of a previous study conducted by Furian et al. (2016) in Brazil.

MLST is a genotyping method appropriate for phylogenetic analysis. In this study, the largest group (56.8%) was clonal complex 13, and it comprised ST13 (13 strains) and ST44 (eight strains). ST13 (7-11-9-10-4-7-8) and ST44 (7-23-9-10-4-7-8) differed in only one of the seven alleles. Generally, predominant STs differ according to geographic location. ST122 was the predominant type in Pakistan and Thailand (Moustafa et al., 2013), while ST3, ST11, and ST62 were predominant in Spain (García-Alvarez et al., 2017). These types were not observed in this study. However, the predominant type in this study, ST13, has been reported in porcine isolates from Denmark (Pors et al., 2011).

In this study, MLST detected low levels of diversity, with six different STs in 37 tetracycline-resistant *P. multocida*. However, PFGE showed 29 different patterns and 25 clusters with 80% similarity. Although some strains with same STs (ST13, ST286, ST44) showed identical or closely related PFGE patterns, most strains with the same ST showed different PFGE patterns. For example, 13 ST13 strains showed ten different PFGE patterns and eight ST44 strains showed six different patterns. By combining both the methods, identical ST types and PFGE patterns were observed in isolates from different farms, suggesting that transmission of antimicrobial-resistant *P. multocida* strains between farms might occur in a geographically discrete population.

In conclusion, several virulence factors were carried by both tetracycline-resistant and tetracycline-susceptible isolates. Results of the genetic analysis and conjugation study suggested that the spread of tetracycline resistance might be due to clonal dissemination rather than horizontal transfer of plasmids. In addition, this study showed the presence of diverse genotypes of tetracycline-resistant *P. multocida*; however, a limited number of clonal lineages (CC13) or recently emerged genotypes (ST44) were also observed. Epidemiological approaches and studies on the development of effective vaccines should focus on major clonal lineages that carried the important virulence factors and frequently observed resistance genes to prevent the transmission of antimicrobial-resistant *P. multocida* strains and control the related diseases.

Conflicts of interest statement

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.001.

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