Investigation of Genetic Diversity of Pasteurella multocida Isolated from Diseased Poultry in Korea

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ABSTRACT

Pasteurella multocida causes fowl cholera which is an economically important disease in poultry industries around the world. In this study, we analyzed the capsular genotype, lipopolysaccharide (LPS) genotype, virulence-associated genes (VAGs) patterns, antimicrobial resistance and genetic diversity in a total of 9 *P. multocida* isolates from poultry with fowl cholera between 2014 and 2019 in Korea. When combining the capsular types with the LPS genotypes, two isolates of the 9 isolates were A:L3, and the others were non-typeable (NT): L3. Of the 23 VAGs, all the isolates harbored *ptfA*, *fimA*, *hsf-1*, *hsf-2*, *pfhA*, *exbB*, *exbD*, *tonB*, *hgbA*, *hgbB*, *fur*, *sodA*, *sodC*, *pmHAS*, *ompA*, *ompH*, *oma87*, *plpB*, *psl*, and *nanH*, whereas *toxA* gene was not detected in any of the 9 isolates. In addition, among the 11 antimicrobials, most of the isolates except for one isolate resistant to florfenicol, exhibited susceptibility to all the antimicrobials. Multi-locus sequence typing (MLST) analysis revealed 5 different sequence types (ST): ST8, ST351, ST352, ST353, and ST368. The ST351, ST352, ST353, and ST368 were identified for the first time in this study, and ST352 and ST353 isolates were largely prevalent nationwide. These STs isolates should be monitored continuously because in some cases, ST352 and ST353 isolates demonstrated high mortality rates. Although only limited numbers of isolates have been analyzed, our findings provide overall characteristics and epidemiological information of the *P. multocida* strains recently prevalent in Korea.

INTRODUCTION

Fowl cholera is an acute and fatal septicemic disease that can affect all types of birds and causes significant economic losses in poultry industries around the world. *Pasteurella multocida* (*P. multocida*) has been reported to be the causal agent of fowl cholera (Li *et al.*, 2018). The disease can range from acute septicemia to chronic and localized infections with wattles, sinuses, leg or wing joints, and foot pads often enlarged, and mortalities have been reported up to 20% (Swayne, 2020).

P. multocida is classified into 16 somatic (Heddleston) serovars (1-16) based on lipopolysaccharide (LPS) antigens and can be divided into five capsular serovars (A, B, D, E, and F) based on capsular antigens. Capsular type A is the most prevalent serovar in cases of fowl cholera (Swayne, 2020), although other capsular types are occasionally incriminated, particularly type F in turkeys, and in some avian strains, non-capsular types have been reported (Davies *et al.*, 2003). In Korea, previous studies suggest that capsular type A has been prevalent in fowl cholera (Kwon & Kang, 2003; Woo & Kim, 2006; Kim *et al.*, 2011); however, these studies provide information only before 2006,

but there is no information on the capsular types, somatic types, and other characteristics of *P. multocida* isolates in the fowl cholera cases that occurred after that period of time. *P. multocida* is reported to have various virulence factors that play an important role in the pathogenesis (Li *et al.*, 2018). The major virulence factors involved in causing fowl cholera include capsular types (Swayne, 2020). Apart from the capsule, many other virulence-associated genes (VAGs) may be related, such as iron acquisition proteins, fimbriae, filamentous haemagglutinin, and sialic acid uptake (Wilkie *et al.*, 2012). The investigation of virulence factors of *P. multocida* may be useful to understand the pathogenesis, epidemiology, and protective immunity which may lead to the development of vaccines against fowl cholera (Tomich *et al.*, 2007). However, the virulence factors of *P. multocida* isolated from poultry have not been investigated in Korea until now.

Control of fowl cholera is primarily ensured by disinfection management and antibacterial therapy has been used extensively in the treatment of infected individuals (Swayne, 2020). However, the prolonged and indiscriminated use of antimicrobials has resulted in an increased incidence of antimicrobial resistance and multi-drug resistant (MDR) isolates of *P. multocida*. This has reduced the efficacy of the antimicrobial agents that are currently used in the treatment of infections in poultry infected with *P. multocida* (Mohamed *et al.*, 2012). Antimicrobial susceptibility tests can provide information about the selection of appropriate antimicrobials and curtail imprudent use of antimicrobials (Regassa & Mohammed, 2019).

Various nucleic-acid-based typing methods have been used to differentiate avian strains of *P. multocida*. The advantage of these methods is that all strains can be typed without depending on phenotypic properties, and the discriminatory power is generally high (Swayne, 2020). Pulsed-field gel electrophoresis (PFGE) has been used to obtain knowledge about routes of transmission and about strain diversity in outbreak cases, but more recently, multi-locus sequence type analysis (MLST) has been used to divide *P. multocida* isolates into genetic types because of its higher reproducibility and discriminatory power (Li *et al.*, 2018). Therefore, identification of serogroups and molecular characteristics of *P. multocida* isolates are important for the epidemiological understanding.

The distribution and prevalence of serovars, pathogenicity, and antimicrobial resistance profiles can vary significantly according to the time and geographical area (Mohamed *et al.*, 2012). However,

there are only a few research reports on *P. multocida* isolated from poultry in Korea between 2000 and 2006 (Kwon & Kang, 2003; Woo & Kim, 2006; Kim *et al.*, 2011) and according to the statistics of Korean Animal Health Integrated System (KAHIS), 22 cases of fowl cholera have occurred in Korea since 2006 (https://www.kahis.go.kr). Hence, the latest information about the characteristics of domestic isolates of *P. multocida* is necessary to conduct appropriate prevention schemes of fowl cholera in Korea. Although the outbreak of fowl cholera in Korea has occurred less compared to other countries (Swayne, 2020) and the number of isolates is few in number, this study was conducted to provide baseline information for the characteristic analysis of *P. multocida*. In addition, we analyzed extensive virulence-associated gene profiles and antimicrobial-resistance patterns of non-capsulated *P. multocida* isolates that have been largely prevalent between 2014 and 2019 in Korea and revealed the epidemiological correlation among these isolates by using MLST.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

A total of 9 avian isolates of *P.multocida* were used in this study. The isolates were collected from cases of fowl cholera in poultry in 5 provinces of Korea between 2014 and 2019, and the host, age, clinical signs, macroscopic lesions and the mortality rate for each case are shown in Table 1. The isolates were cultured on blood agar (Synergy Innovation, Seongnam, Korea) and MacConkey agar (Becton Dickinson, Sparks, MD, USA) in the air at 37°C for 24 h. Suspected colonies (mucoid and non-haemolytic) were identified by Gram staining and biochemical identification using the VITEK II system (BioMérieux, Marcy l'Etoile, France) according to the previous methods (Pujiono *et al.*, 2018). For further confirmation, PCR assay was performed with primers specific for the amplification of the KMT1 gene (Townsend *et al.*, 1998). The isolates were stored at -80°C in 30% [v/v] glycerol in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) until further use. Genomic DNA was extracted from colonies grown on blood agar plates using the commercial kit DNeasy Blood and Tissue (Qiagen, Hilden, Germany).

Capsular typing and LPS genotyping

The capsular types of the isolates were confirmed by multiplex capsule PCR typing with the capsule-specific primer pairs specific for *capA*, *capB*, *capD*, *capE*, and *capF* as described by Townsend *et al.* (Townsend *et al.*, 2001). The *P. multocida* reference strains ATCC 43137

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Table 1 – Characteristics and epidemiological sources of 9 *P. multocida* isolates.

Strain	Year	Region	Host	Age (weeks)	Clinic status	Macroscopic lesion	Mortality rate (%)
14PM1	2014	Jeonam	Layer chicken	50	Depression, white diarrhea, sudden death	Enlargement and multiple necrotic foci of the liver, salpingitis, peritonitis	3.3
14PM2	2014	Jeonbuk	Penkin duck	50	Sudden death without previous signs	Enlargement and multiple necrotic foci of the liver, enlargement of spleen	0.19
17PM1	2017	Gyeongnam	Muscovy duck	19	Sudden surge in mortality without previous signs (6.1%/day)	Fibrinous pericarditis, enlargement and multiple necrotic foci of the liver	21.5
17PM2	2017	Jeonam	Penkin duck	5	Lameness, sudden death	Fibrinous pericarditis, enlargement of liver, enlargement and caseous exudates of hock joint	Unknown
18PM1	2018	Gyeongnam	Korean native chicken	12	Sudden death without previous signs	Enlargement and multiple necrotic foci of the liver	0.3
18PM2	2018	Gyeongnam	Korean native chicken	12	Sudden death without previous signs	Fibrinous pericarditis, enlargement and multiple necrotic foci of the liver	0.25
18PM3	2018	Jeonam	Layer chicken	28	Depression, green diarrhea	Fibrinous perihepatitis, air sacculitis	10
18PM4	2018	Gyeonbuk	Penkin duck	12	Sudden death previous signs	Enlargement and multiple necrotic foci of the liver	0.25
19PM1	2019	Gangwon	Layer chicken	26	Sudden death without previous signs (0.3%/day)	Enlargement and multiple necrotic foci of the liver	1.3

(*capA*), ATCC 43017 (*capB*), NCTC 10325 (*capD*), NCTC 10326 (*capE*), and the *P. multocida* clinical isolate from porcine (*capF*) (Kim *et al.*, 2019) were used as positive control for the genes indicated. Recently, Harper *et al.* (2015) replaced conventional somatic serotyping with the genotyping of LPS antigens by developing a multiplex PCR by targeting the genes encoding the LPS structures (LPS-mPCR). The LPS-mPCR assay was performed as previously described and the isolates were classified into one of the eight LPS types (termed L1-L8) (Harper *et al.*, 2015). The *P. multocida* strain X-73 (L1), M-1404 (L2), P-1059 (L3), P2192 (L4), P-2095 (L5), P-2100 (L6), P-1591 (L7), and P-2723 (L8) were used as positive control for the genes (L1-L8).

Transmission electron microscopy (TEM) to identify the presence of capsules

For morphological analysis of the capsule-presence of the genetically non-typable isolates, bacterial cultures were harvested and washed once with phosphate-buffered saline (0.01M, pH 7.2). Then they were suspended in cacodylate buffer (0.1M, pH 7.0) containing 5% glutaraldehyde and 0.15% (wt/vol) ruthenium red (TCI America, Tokyo, Japan), as described previously (Jacques & Foiry, 1987). Fixed cells were suspended in cacodylate buffer and treated with the polycationic ferritin (Sigma Chemical Co., St. Louis, Mo, USA), final concentration, 1.0 mg/ml, for 30 min at 20°C (Weiss *et al.*, 1979). The reaction was stopped

by 10-fold dilution, and the samples were centrifuged and washed three times in cacodylate buffer. They were then immobilized in 4% agar, washed three times in cacodylate buffer, and postfixed with 2% osmium tetroxide for 2 h. The postfixed samples were washed three times in cacodylate buffer and dehydrated through a series of acetone washes. After dehydration in propylene oxide, samples were embedded in Spurr low-viscosity embedding resin. Thin sections were stained with uranyl acetate and lead citrate and visualized by transmission electron microscope at an accelerating voltage of 60 kV, as described previously (Jacques & Foiry, 1987).

Detection of virulence-associated genes (VAGs) by PCR

Twenty-three pairs of oligonucleotide primers were used for the detection of VAGs including genes encoding structures: adhesin factors (*ptfA*, *fimA*, *hsf-1*, *hsf-2*, *pfhA* and *tadD*), toxin (*toxA*), iron acquisition factors (*exbB*, *exbD*, *tonB*, *hgbA*, *hgbB*, and *fur*), superoxide dismutases (*sodA* and *sodC*), sialidases (*nanB* and *nanH*), hyaluronidase (*pmHAS*), and membrane and porin proteins (*ompA*, *ompH*, *oma87*, *plpB*, and *psl*) according to previous studies (Kasten *et al.*, 1997; Ewers *et al.*, 2006; Atashpaz *et al.*, 2009; Aski & Tabatabaei, 2016). The details of base sequences, the size of amplified products for the primers, and annealing temperature are listed in Table 2. PCR amplification was performed using a Mastercycler ep Gradient S

Table 2 – The size of amplified products for the primers and annealing temperature used for the detection of virulence-associated genes in *P. multocida* isolates.

Gene function	gene	Description	Size (bp)	Annealing temperature (ºC)	Reference
Adhesins	*ptfA*	type 4 fibriae	488	55	Aski & Tabatabaei (2016)
	fimA	Fimbriae	866	55	Aski & Tabatabaei (2016)
	hsf-1	Autotransporter adhesion	654	49	Aski & Tabatabaei (2016)
	hsf-2	Autotransporter adhesion	433	55	Aski & Tabatabaei (2016)
	pfhA	Filamentous hemagglutinin	286	55	Aski & Tabatabaei (2016)
	tadD	Putative nonspecific tight adherence protein D	416	55	Aski & Tabatabaei (2016)
Toxin	*toxA*	Dermonecrotic toxin	864	55	Aski & Tabatabaei (2016)
Iron acquisition	*exbB*	Accessory protein Ton-dependent transport of iron	283	55	Aski & Tabatabaei (2016)
	exbD	Accessory protein Ton-dependent transport of the iron compound	247	55	Aski & Tabatabaei (2016)
	tonB	Iron transporters, transport ferric-siderophore complexes	261	55	Aski & Tabatabaei (2016)
	hgbA	Hemoglobin-binding protein	267	55	Aski & Tabatabaei (2016)
	hgbB	Hemoglobin-binding protein	419	54	Atashpaz *et al.* (2009)
	fur	Ferric uptake regulation protein	244	54	Aski & Tabatabaei (2016)
Superoxide dismutases	*sodA*	Superoxide dismutases	361	55	Ewers *et al.* (2006)
	sodC	Superoxide dismutases	235	55	Ewers *et al.* (2006)
Sialidases	*nanB*	Outer membrane-associated proteins, an autotransporter protein	555	55	Aski & Tabatabaei (2016)
	nanH	Outer membrane-associated proteins, small sialidases	287	55	Aski & Tabatabaei (2016)
Hyaluronidase	*pmHAS*	Hyaluronan synthase	430	52.5	Aski & Tabatabaei (2016)
Outer membrane and porin proteins	*ompA*	Outer membrane protein A	201	55	Aski & Tabatabaei (2016)
	ompH	Outer membrane protein H	438	55	Aski & Tabatabaei (2016)
	oma87	Outer membrane protein 87	838	55	Aski & Tabatabaei (2016)
	plpB	Lipoprotein B	282	55	Aski & Tabatabaei (2016)
	psl	Porin protein	470	50	Kasten *et al.* (1997)

(Eppendorf, Hamburg, Germany), and amplification products were analyzed by gel electrophoresis on a 1.5% agarose gel (SeaKem®LE Agarose, FMC Bioproducts, Rockland, MA, USA) using the mini-gel electrophoresis system (Mupid-exu, Mupid, Tokyo, Japan). The gel was scanned with Gel Doc XR (Bio-Rad, Hercules, CA, USA). The positive PCR products were purified using Qiagen purification kits (Qiagen, Hilden, Germany) and sent for sequencing (Macrogen, South Korea) in order to confirm their sequences. After forward and reverse sequences were aligned, sequence analysis and comparison with relevant sequences were performed using the BLAST program (National Center for Biotechnology Information, Bethesda, Maryland, USA).

Antibiotic susceptibility test

The minimum inhibitory concentrations (MICs) for 9 *P. multocida* isolates was determined by the broth microdilution method using Sensititre panel CMV3AGNF and BOPO6F (Trek Diagnostic Systems, East Grinstead, UK), according to the protocols of the manufacturer protocols. The 11 antimicrobials tested included amoxicillin/clavulnic acid, ampicillin, ceftiofur, enrofloxacin, florfenicol, gentamicin, spectinomycin, sulfisoxazole, tetracycline, tilmicosin and trimethoprim/sulphamethoxazole. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. The breakpoints of MIC were based on the Clinical and Laboratory Standards Institute document M21-A2 (CLSI, 2002).

Multi-locus sequence typing (MLST) analysis

MLST based on the seven housekeeping genes (*adk*, *est*, *pmi*, *zwf*, *mdh*, *gdh*, and *pgi*) was performed on all 9 isolates using primers and protocols described in RIRDC MLST Database (https://pubmlst.org/pmultocida/). The RIRDC scheme was developed by Sounthi Subaaharan and Pat Blackall (Subaaharan *et al.*, 2010). Amplification products were sequenced at Macrogen (Seoul, Korea). The sequence of each locus was checked on the PubMLST RIRDC Database website for the determination of the allele and sequencing type (ST). All STs have been deposited in the MLST database. Dendrogram for visualizing genomic relations among 9 isolates was generated by the Mega-X software based on the joint analysis of seven housekeeping

genes (Applied Maths, Sint-Martens-Latem, Belgium). Additionally, STs were grouped into clonal complexes (CC) based on their similarity to six out of seven alleles using goeBURST (http://goeburst.phyloviz.net).

RESULTS

Distribution of capsular types and LPS genotypes

The capsular types of the 9 isolates were determined by capsular PCR typing and it was confirmed that two isolates (18PM2 and 19PM1) were capsular type A, but the others were non-typable (Supplementary figure S1). Electron microscopic examination of non-typable isolates indicated that they were non-capsulated. The transmission electron microscopy (TEM) image of cells of 17PM1, a representative non-capsulated isolate, was presented in figure 1A and these cells were not covered with a layer of ferritin granules. However, in 18PM2 and 19PM1 identified as capsular type A by capsular PCR typing, the ferritin-labeled capsular materials surrounded the cells completely (Figure 1B and 1C). Regarding the LPS genotype, all the isolates were of genotype L3 (Supplementary figure S2).
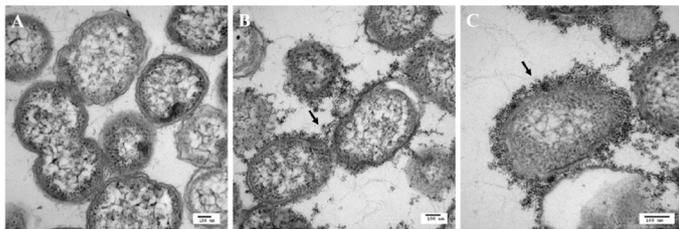

Figure 1 – Transmission electron microscopy (TEM) images of thin sections of *P. multocida* 17PM1 (capsular non-typeable) (A), 18PM2 (capsular type A) (B), and 19PM1 (capsular type A) (c) cells labeled with polycationic ferritin. Arrows show capsular materials. (A) magnification 6000x, scale bar = 100 nm; (B) magnification 6000x, scale bar = 100 nm; (C) magnification 100000x, scale bar = 100 nm.

Distribution of VAGs

The distribution of 23 VAGs for *P. multocida* isolates is presented in Figure 2. All the isolates harbored the adhesion encoding genes (including *ptfA*, *fimA*, hsf-

1, *hsf-2*, and *pfhA*), iron acquisition factor genes (*exbB*, *exbD*, *tonB*, *hgbA*, *hgbB*, and *fur*), various outer membrane proteins encoding genes (*pmHAS*, *ompA*, *ompH*, *oma87*, *plpB*, and *psl*), Superoxidae dismutase encoding genes (*sodA* and *sodC*) and sialidase encoding gene (*nanH*). Most of the isolates except for 19PM1 (88.9%) harbored the sialidase encoding gene, *nanB*. However, dermonecrotic toxin, a toxA gene that encodes the most important virulence factor of *P. multocida*, was not detected in any of them (Supplementary figure S3). The *tadD* gene, an adhesion encoding gene, was exclusively detected in 14PM1 of the 9 isolates (11.1%).

Antimicrobial susceptibility

The range of antimicrobial MICs, MIC_{50}, and MIC_{90} of the *P. multocida* isolates are represented in Table 3. Taken overall, the isolates showed quite a uniform sensitivity. All the isolates except for one isolate (14PM2) displayed sensitivity to the tested antibiotics, and 14PM2 showed resistance to florfenicol (>8 µg/ml). MIC_{50} and MIC_{90} values for ceftiofur and trimethoprim/sulphamethoxazole were the lowest of all the antibiotics tested: ≤0.12 µg/ml and ≤0.12 µg/ml for 9 isolates. Higher MIC_{50} and MIC_{90} values were observed for spectinomycin (8 µg/ml, 16 µg/ml) and sulfisoxazole (≤16 µg/ml, 32 µg/ml). For florfenicol, quite a large gap of concentrations between MIC_{50} (0.5 µg/ml) and MIC_{90} (>8 µg/ml) were found. In this case, only 14PM2 isolate showed the highest MIC value (>8 µg/ml) for florfenicol and remaining 8 isolates displayed lower MIC values (0.5 µg/ml) under breakpoints.

MLST

MLST analysis of the 9 *P. multocida* isolates revealed 5 different STs: ST8 (1/9), ST351 (1/9), ST352 (3/9), ST353 (3/9), and ST368 (1/9). The ST351 (*adk*4-*est*5-*pmi*23-*zwf*1-*mdh*5-*gdh*6-*pgi*14), ST352 (*adk*53-*est*86-*pmi*23-*zwf*58-*mdh*42-*gdh*58-*pgi*85), ST353 (*adk*53-*est*86-*pmi*23-*zwf*1-*mdh*42-*gdh*58-*pgi*85) and ST368

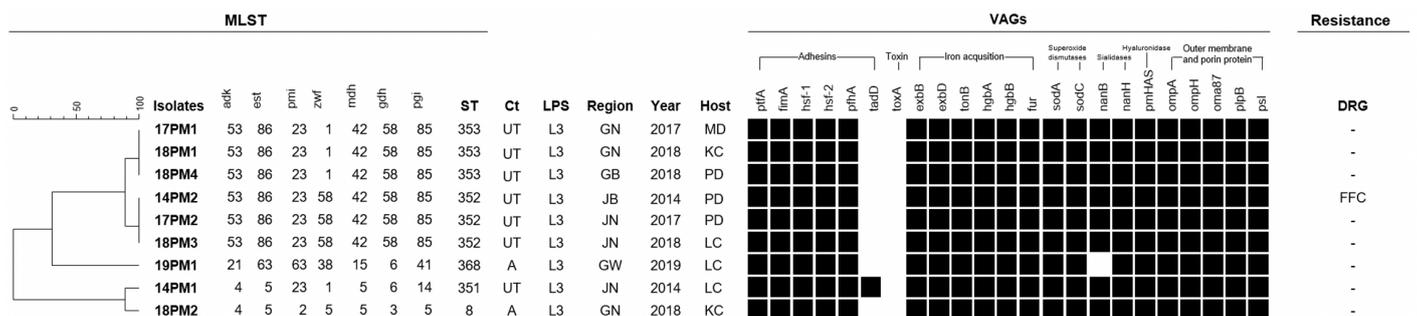| MLST														VAGs																								Resistance
														Adhesins					Toxin		Iron acqusition						Superoxide dismutases		Sialidases		Hyaluronidase	Outer membrane and porin protein							
	adk	est	pmi	zwf	mdh	gdh	pgi	Isolates	ST	Ct	LPS	Region	Year	Host	ptfA	fimA	hsf-1	hsf-2	pfhA	tadD	toxA	exbB	exbD	tonB	hgbA	hgbB	fur	sodA	sodC	nanB	nanH	pmHAS	ompA	ompH	oma87	plpB	psl	DRG
	53	86	23	1	42	58	85	17PM1	353	UT	L3	GN	2017	MD	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-
	53	86	23	1	42	58	85	18PM1	353	UT	L3	GN	2018	KC	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-
	53	86	23	1	42	58	85	18PM4	353	UT	L3	GB	2018	PD	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-
	53	86	23	58	42	58	85	14PM2	352	UT	L3	JB	2014	PD	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	FFC
	53	86	23	58	42	58	85	17PM2	352	UT	L3	JN	2017	PD	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-
	53	86	23	58	42	58	85	18PM3	352	UT	L3	LC	2018	LC	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-
	21	63	63	38	15	6	41	19PM1	368	A	L3	GW	2019	LC	■	■	■	■	■			■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	-
	4	5	23	1	5	6	14	14PM1	351	UT	L3	JN	2014	LC	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-
	4	5	2	5	5	3	5	18PM2	8	A	L3	GN	2018	KC	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-

Figure 2 – Dendrogram representing correlation among the 9 *P. multocida* isolates according to biological and genetic characteristics. Phylogenetic tree based on the 7 concatenated gene sequences. Black square: positive reaction; white squares: negative reaction; ST: sequence type; Ct: capsular type; GN: Gyeongnam; GB: Gyeongbuk; JN: Jeonam; JB: Jeonbuk; GW: Gangwon; MD: Muscovy duck; KC: Korean native chicken; PD: Pekin duck; LC: Layer chicken; FFC: florfenicol.

Table 3 – MICs for 11 antimicrobial agents for 9 *P. multocida* isolates.

Antimicrobial	Breakpoint for resistance (µg/mL) [1]	Resistance (%)	MIC Ranges	MIC$_{50}$ (µg/mL) [2]	MIC$_{90}$ (µg/mL) [3]
Amoxicillin/ Clavulanic acid	≥32	0	≤1	≤1	≤1
Ampicillin	≥32	0	≤1	≤1	≤1
Ceftiofur	≥8	0	≤0.12	≤0.12	≤0.12
Enrofloxacin	≥2	0	≤0.12-0.25	≤0.12	0.25
Florfenicol	≥8	11	0.5->8	0.5	>8
Gentamicin	≥16	0	0.5-2	1	2
Spectinomycin	≥128	0	8-16	8	16
Sulfisoxazole	≥512	0	≤16-128	≤16	32
Tetracycline	≥16	0	≤4	≤4	≤4
Tilmicosin	≥32	0	≤4	≤4	≤4
Trimethoprim/ sulphamethoxazole	≥4	0	≤0.12	≤0.12	≤0.12

[1] The MIC breakpoint, The breakpoints were obtained from Clinical and Laboratory Standards Institute (CLSI) document M31-A2 (CLSI, 2002) and previous study (Huang et al., 2009).

[2] MIC$_{50}$, antimicrobial concentrations at which 50% of the isolates were inhibited.

[3] MIC$_{90}$, antimicrobial concentrations at which 90% of the isolates were inhibited.

(*adk*21-*est*63-*pmi*63-*zwf*38-*mdh*15-*gdh*6-*pgi*41) were identified for the first time in this study. ST352 and ST353 have *zwf*58 and *zwf*1, respectively, which have a C/T mutation at position 475 of *zwf* locus. The isolates of ST8 and ST368 were capsular type A, whereas the isolates of ST351, ST352 and ST353 were non-capsulated (Figure 2). According to the goeBURST, the 9 isolates were divided into 4 CCs (CC8, CC351, CC352, and CC368). The 6 isolates including ST352 and ST353 were a single CC (CC352). The remaining 3 isolates including ST8, ST351, and ST368 were singletons (CC8, CC351, and CC368, respectively).

DISCUSSION

Fowl cholera, caused by *P. multocida*, is a highly contagious disease occurring sporadically or enzootically in most of the countries worldwide. In Korea, there are just two research papers on fowl cholera of domestic chickens in Gyeong-gi Province in 2006, and until date, there has been no recent data on fowl cholera (Woo & Kim, 2006; Kim *et al.*, 2011). Therefore, following a need to analyze the epidemiological patterns and characteristics of the recent *P. multocida* isolates in Korea, we confirmed the capsular type and LPS type of *P. multocida* isolated from various poultry breeds with fowl cholera in the southern and northeast part of Korea from 2014 to 2019. In addition, we have evaluated the antimicrobial susceptibility of *P. multocida* isolates and detected virulence-associated genes. Furthermore, MLST was used to analyze the genetic diversity and molecular epidemiology of the isolates in Korea.

In Korea, previous studies have shown that all isolates expressed capsular type A (Kwon & Kang, 2003; Woo &

Kim, 2006; Kim *et al.*, 2011). Aski & Tabatabaei, 2016, Li *et al.* (2018) and Shivachandra *et al.* (2005) reported that most of the poultry isolates from Iran, China, and India were of capsular type A. However, non-capsulated *P. multocida* isolates from poultry have been identified in the United States, United Kingdom, and Indonesia (Rhoades & Rimler, 1987; Mariana & Hirst, 2000; Davies *et al.*, 2003). In the present study, 18PM2 and 19PM1 of the 9 isolates were identified as capsular type A, whereas seven isolates were not identified as any capsular type. This case with a larger proportion of non-capsulated isolates was rare compared to the previous studies (Rhoades & Rimler, 1987; Mariana & Hirst, 2000; Aski & Tabatabaei, 2016). It might be because the number of samples is too small, as we only tested nine strains. Therefore, more monitoring and investigation are needed to identify which types of *P. multocida* are prevalent in the future. It has been known that capsulated isolates are more virulent than the non-capsular variants (Heddleston *et al.*, 1964). However, in this study, of seven farms from where non-capsular strains were isolated, three farms of Pekin duck and Korean native chicken showed very low mortality rates (less than 0.5%) in their flocks; whereas the farms of Muscovy duck and layer chicken showed the high mortality rates (about 10 to 21.5%) in the flocks. It is assumed that other complex and variable factors such as host species, farming environment, and characteristics of the strain itself probably affect the pathogenicity of *P. multocida*.

There are two assay methods for LPS types of *P. multocida*: Heddleston serotyping and LPS genotyping (Harper *et al.*, 2015). Heddleston serotyping has been used in the past to identify serovars based on

the traditional method; currently, it is replaced by the developed LPS PCR. Turni *et al.* (2018) pointed out that the Heddleston serotyping scheme is not suitable for *P. multocida*, stating that the traditional serotyping methodology may be inaccurate. The LPS PCR has been used to detect the LPS genotype of *P. multocida* isolates in China, and in 2016, Wang *et al.* reported that avian origin *P. multocida* isolates were mainly in L1 (Wang *et al.*, 2016). However, in this study, all the isolates were found to express LPS genotype L3. In Australia, the dominant LPS genotype of *P. multocida* isolates from diseased poultry was also L3 (Turni *et al.*, 2018). The lipopolysaccharide produced by strains belonging to the L3 LPS genotype contains outer core structures similar to the oligosaccharide component of P, Pk, and Forssman antigen, found on the surfaces of many cell types in vertebrates, and so the expression of L3 molecules may help bacteria to survive in vivo by avoiding the host immune system (Harper *et al.*, 2013). Peng *et al.* (2018) speculated that this mechanism of L3 backs up why L3 genotypes are largely prevalent in *P. multocida* isolates from diseased animals. Most of the LPS genotype L3 isolates are in agreement with serovar 3 or 4 as a result of the comparison between the Heddleston serotyping and LPS genotyping (Harper *et al.*, 2015). Applying these findings to previous studies in Korea, the isolates, Heddleston type 1, 10X11 and 1X12X14 can be considered as L1 or L6 (Kwon & Kang, 2003; Woo & Kim, 2006; Kim *et al.*, 2011). In summary, nine *P. multocida* isolated in this study were identified as A:L3 and NT (non-typable):L3 and the isolates in the previous studies were A:L1 or A:L6. Apparently, recently prevalent isolates are presumed to be inconsistent with the isolates identified before 2006 in Korea.

The pathogenicity of *P. multocida* is reported to be associated with various virulence factors and the wide distribution of VAGs is significant for the survival of *P. multocida* in the host environment (Aski & Tabatabaei, 2016). In addition, Tang *et al.* (2009) provided the new epidemiological information on the prevalence and distribution of virulence factors of *P. multocida* isolates. Kim *et al.* (2019), Awad & Abd El-Hamid (2019), and Aski & Tabatabaei (2016) found that the prevalence of some VAGs encoding adhesin factors (*ptfA*, *fimA*, *hsf-1*, *hsf-2*, *pfhA*, *tadD*), toxin (*toxA*), sialidase factors (*nanB*, *nanH*), and outer membrane proteins (*ompA*, *ompH*, *oma87*, *plpB*, *psl*) in *P. multocida* isolates from diseased swine, rabbits, poultry and other animals. We also investigated the presence of 23 virulence genes in 9 isolates in this study. All the isolates harbored

ptfA, *exbB*, *hgbA*, *sodA*, *sodC*, *oma87*, *ompA*, and *plpB*. These results are in agreement with those of the previous study by Aski & Tabatabaei (2016). The equal and similar frequencies of *oma87* (100%), *plpB* (98%), *ptfA* (96%), and *hgbA* (98%) were stated by Furian *et al.* (2016). However, none of the isolates possessed *toxA* in this study and Furian *et al.* (2016) and Aski & Tabatabaei (2016) also reported the absence of *toxA* in all the poultry isolates. According to the previous studies, the *toxA* gene is significantly associated with the capsular type D strains (Ewers *et al.*, 2006; Tang *et al.*, 2009; Aski & Tabatabaei, 2016) and its related toxin, dermonecrotic toxin plays an important role in the pathogenesis of progressive atrophic rhinitis in pigs (Pullinger *et al.*, 2004). Herein, *tadD* which is significantly related to capsular type A was detected in only 14PM1 (non-capsulated), whereas Furian *et al.* (2016) and Aski & Tabatabaei (2016) suggested that 38% and 60% of the poultry isolates were positive for the *tadD*, respectively. This difference may relate that the most common isolates in the present study were non-capsulated; whereas the isolates from the previous studies were almost capsular type A. Although we could not grasp the correlation between capsular type and VAGs as most of them were non-capsulated, the wide distribution of VAGs detected in our isolates indicates their potential role contributing to pathogenicity of *P. multocida*.

Antibiotic therapy is still considered as a tool in the treatment of fowl cholera. However, antimicrobial resistance has become a global problem as resistant isolates have emerged by the excessive and unjustified use of antimicrobials (Regassa & Mohammed, 2019). Fortunately, in this study, all the isolates except for 14PM2 which was only resistant to florfenicol were susceptible to 11 antimicrobials. However, in the neighboring country, China, the prevalence of *P. multocida* isolate resistant to veterinary antibiotics including florfenicol, doxycycline, tetracycline, and sulfamethazine was found to be more than 46.7% (Li *et al.*, 2018). Similarly, resistance to tetracycline and amoxicillin has been reported to be 100% in Egypt (Mohamed *et al.*, 2012). It has been reported in Korea, all isolates displayed resistance to streptomycin and some isolates were resistant to gentamicin and tetracycline (Woo & Kim, 2006; Kim *et al.*, 2011). The resistance of *P. multocida* isolates to antimicrobials varies depending on the regions and classes of used antimicrobials. High susceptibility to most of the antimicrobials in this study may result from two possibilities: in our cases with low mortality, antibiotics might be hardly prescribed in the

field because most of the flocks had few symptoms, and in high mortality-cases in layer chicken, use of the antibiotics might be limited due to presence of antimicrobial residues in the eggs.

To reveal the genetic correlation in the isolates with identical capsular and LPS types, MLST was further analyzed. Of the 9 isolates, seven were NT:L3. These isolates were ST351 (1), ST352 (3), and ST353 (3), which were new STs. Of these isolates, ST 352 and ST353 isolates were included in a single CC (CC352) and showed the same patterns of VAGs distribution. The wide-spread nature of ST352 and ST353 isolates in the southern part of Korea may be attributed to the fact that farmers and veterinarians have not identified the disease at an early stage as most of the farms infected with these STs isolates showed few clinical signs and low mortality in their flocks. However, in some cases, ST352 and ST353 isolates from layer chicken and muscovy duck demonstrated high mortality rates; it is believed that these STs isolates can become increasingly prevalent and cause high mortality in the vulnerable species, thereby leading to significant economic losses in the near future. Therefore, we recommend continuous surveillance of these STs outbreak and monitoring the spread of *P. multocida* in poultry farms. Two isolates, A:L3, were identified as ST8 and ST368 respectively, and of these, ST 368 was identified as a new ST. The ST368 isolate was isolated in the northeast part of Korea (Gangwon-do), far away from other provinces and was genetically heterogeneous with ST352 and ST353. The distribution patterns of VAGs were also different from these STs. The ST8 isolate has detected in the southern part of Korea and has been reported to be prevalent in China and Australia (Blackall *et al.*, 1998; Singh *et al.*, 2013; Li *et al.*, 2018). The ST8 isolates were reported to cause serious fowl cholera in Australia (Blackall *et al.*, 1998; Singh *et al.*, 2013), but the mortality rate was less than 1% in the farm where our ST8 isolate was detected.

The isolates we analyzed showed high susceptibility to most antimicrobials. However, the continued use of antibiotics can cause the increased incidence of antibiotic resistance and MDR forms of *P. multocida* in the near future. Therefore, strategies should be established in a direction that fowl cholera can be prevented before cure. In addition, in the future, it is required to develop or manufacture the vaccine against isolates which are currently prevalent in Korea. ST352 and ST353 isolates could be a potential risk factor in specific species (layer chicken and Muscovy duck). The survey of characteristics of *P. multocida*

isolates, including their STs analysis and VAGs patterns should be continuously conducted to tract the spread of fowl cholera in poultry farms. The distribution and prevalence of serotypes, virulence genes, and MLST profiles analyzed in this study will function as a basis for prevention and control programs.

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Supplementary Figures