Characterisation of *Pasteurella multocida* isolates from pigs with pneumonia in Korea

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**Abstract**

**Background:** *Pasteurella multocida* is responsible for significant economic losses in pigs worldwide. In clinically diseased pigs, most *P. multocida* isolates are characterised as subspecies *multocida*, biovar 2 or 3 and capsular type A or D; however, there is little information regarding subspecies, biovars, and other capsular types of *P. multocida* isolates in Korea. Here, we provided information covering an extended time period regarding *P. multocida* in pigs with pneumonia in Korea using phenotypic and genotypic characterisations and data associated with the minimum inhibitory concentrations.

**Results:** The overall prevalence of *P. multocida* between 2008 and 2016 was 16.8% (240/1430), with 85% of the *P. multocida* isolates (204/240) coinfected with other respiratory pathogens. Of the 240 isolates, 166 were included in this study; all of these *P. multocida* isolates were characterised as subspecies *multocida* and the most prevalent phenotypes were represented by biovar 3 (68.7%; n = 114) and capsular type A (69.9%; n = 116). Additionally, three capsular type F isolates were identified, with this representing the first report of such isolates in Korea. All biovar 1 and 2 isolates were capsular types F and A, respectively. The virulence-associated gene distribution was variable; all capsular type A and D isolates harboured *pmHAS* and *hsf-1*, respectively (P < 0.001), with type F (biovar 1) significantly correlated with *hsf-1* (P < 0.05) and *pfbA* (P < 0.01), biovar 2 highly associated with *pfbA* and *pmHAS*, and biovar 3 significantly correlated with *hsf-1*, *pmHAS*, and *hgbB* (P < 0.001), whereas biovar 13 was related only to *hgbB* (P < 0.05). The highest resistance rate was found to be to oxytetracycline (63.3%), followed by florfenicol (16.3%).

**Conclusions:** *P. multocida* subspecies *multocida*, biovar 3, and capsular type A was the most prevalent isolate in this study, and our findings indicated the emergence of capsular type F in Korea. Moreover, prudent use of oxytetracycline and florfenicol is required because of the identified high resistance rates. Further studies are required for continuous monitoring of the antimicrobial resistance, prevalence, and epidemiological characterisation of *P. multocida*, and experimental infection models are needed to define the pathogenicity of capsular type F.

**Keywords:** Antimicrobial resistance, Biovar, Capsular type, *Pasteurella multocida*, Virulence-associated gene

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**Background**

*Pasteurella multocida* (*P. multocida*) is a commensal and opportunistic pathogen of the oral, nasopharyngeal, and upper respiratory tract [1] and the causative agent of a wide range of infections leading to high economic impact [2]. In pigs, *P. multocida* is associated with progressive atrophic rhinitis (PAR), and together with other respiratory pathogens, plays a significant role in porcine respiratory disease complex (PRDC) [3–6]. *P. multocida* prevalence has been reported as 8.0% in diseased pigs with pneumonia or PAR in China, and from 10.3 to 15.6% in pigs with pneumonia in Korea. Additionally, *P. multocida* constitutes 15.6% of isolated respiratory pathogens in the United States [3, 5, 7, 8].

*P. multocida* can be divided into three subspecies (*multocida*, *septica*, and *gallicida*) and 13 biovars (1–10 and 12–14) based on carbohydrate fermentation and production of the ornithine decarboxylase (ODC) enzyme [9–11]. The majority of swine isolates are subspecies *multocida* and mostly assigned as biovars 2 or 3 [1, 10, 12, 13]. Additionally, five capsular types based on capsular antigens (A, B, D, E, and F) have been described in *P. multocida*, with capsular types A, B, D, and F recovered from swine [1, 14]. Capsular types A and D are
| Gene function | Target gene | Description | Sequence (5′ – 3′) | size (bp) | Reference |
|---------------|-------------|-------------|--------------------|----------|-----------|
| Capsule serotypes | kmt1 | Identification of all P. multocida isolates | ATCCGCTATTTACCAGTGGGCTGTAAGAAGCACTGCAGCA | 460 | [19] |
|  | hyaD-hyaC | Serogroup A cap gene | TGCCAAATGACGATGAGGATGG TTGACATTTTGCAGTGTCA | 1044 | [20] |
|  | bcbD | Serogroup B cap gene | CATTATCCAGCTCCACGGCCCGAGAGTTC | 760 | [20] |
|  | dcbF | Serogroup D cap gene | TTACAAAAAGAAGAGGACTGGAGACCACATCTACACCATATACCA | 657 | [20] |
|  | ecbJ | Serogroup E cap gene | TCCGCGAGAAATTATTGAGCTGCTGTGCTTGTTC | 511 | [20] |
|  | fcbF | Serogroup F cap gene | AATCAGGAGACCAGAAATACAG TCCGCGCGAATATTACCTGCT | 851 | [20] |
| Outer membrane and porin proteins | oma87 | Outer membrane protein 87 | ATGAAAAACCTTTTAATTGCGAGC TGACTTGCGCAGTTGCATAAC | 948 | [17] |
|  | ompH | Outer membrane protein H | CGCGTATGAAGGTTAGGT TTAGATTTGTCGAGTCAAC | 438 | [17] |
|  | plpB | Outer membrane protein | TTTGGGTGGTCTGTGTCTCT AGTCATTTTACATTGCTGAG | 282 | [17] |
|  | psl | Porin protein | TCTGATGGCATGAAAAACTAACTAAAGTA AAGGATCCTTAGTATGCTAACACAGGACG | 470 | [17] |
| Adhesins | fimA | Fimbriae | CCATCGGATCTAAACGACCTA AGTATTAGTTCCTGCGGGTG | 866 | [18] |
|  | pIIA | Filamentous haemagglutinin | AGCTGATCTAAGTGGTAGAACTGAG TGGTACATTGGTGAATGCTG | 275 | [21] |
|  | ptfA | Fimbriae | TGTGGAATTCAGCATTTTAGTGTGTCT CATGGAATTTACCTGCTGAG | 488 | [18] |
|  | hsf-1 | Autotransporter adhesion | TTGAGTCTGGCTGTAGATCTG AGCTTTGACGTGGAGCAACCTGCTA | 654 | [18] |
|  | hsf-2 | Autotransporter adhesion | ACCGCAACATCTGATTTAC TGACTGACATCGGATAGTAAC | 433 | [18] |
| Superoxide dismutases | sodA | Superoxide dismutases | TACCAAGATTAGGCTACGC GAAGGGTTGGCCTGCGCT | 361 | [17] |
|  | sodC | Superoxide dismutases | AGTTAGTAGCGGGGTGGCA TGGTGGTGGATGTCATATGG | 235 | [17] |
| Iron acquisition related factor | exbB | Iron regulated and acquisition factors | TTGGGTCGGATGTGAGAGAC TGAGAGAACGGACTAAA | 283 | [18] |
|  | exbBD-tonB | Iron acquisition related factors | GGTGGGTGATTTAGTATGGGC GCACTACAGCTGACGGT | 1144 | [18] |
|  | fur | Iron regulated and acquisition factors | GTTTACCGTGTTTACACCGTA CATTACACATTGCCCTAAC | 244 | [18] |
|  | tpbA | Iron acquisition related factor | TGGTGGAAAGCGTTAAGCGT TAAGCTGATGACGAAAAGCC | 728 | [21] |
|  | hgbA | Haemoglobin binding protein | TGGCGGATAGTGCATCAAG CCAAGAACCACCTACCCA | 419 | [17] |
|  | hgbB | Haemoglobin binding protein | TCCATGTGTCAGCGTGGAC TTTGCGTCGTGACTTACC | 499 | [21] |
| Neuraminidases | nanB | Neuraminidases | GTCTCTATAAAGGTACCCGGA ACAGCAAAGGAAAGACTGCAGC | 554 | [17] |
|  | nanH | Neuraminidases | GAAATTTGGCGGCGAACCT GTTCTCGCTGCTCATCTACT | 360 | [17] |
| Hyaluronidase | pmHAS | Hyaluronidase | TCAATTGTTGCGATAGTCCCGTAG TGGCGGATAGTGCATCAAG | 430 | [17] |
| Toxin | toxA | Dermonecrotic toxin | TTCCATGAGGCGACAAAG GATGCGCACACCCTATAG | 846 | [21] |
most commonly cultured from pulmonary lungs and PAR, respectively, whereas capsular types B and F are rarely isolated from pigs [3, 14–16]. In Korea, numerous studies suggest that capsular type A is more prevalent in porcine pneumonia than type D [7, 8, 15]; however, limited information is available regarding subspecies, biovars, and other capsular types of *P. multocida* isolates in Korea.

*P. multocida* reportedly possesses various virulence factors that play a significant role in pathelorealisation and survival in the host environment [3, 17, 18]. Furthermore, there is a clear correlation between certain virulence factors and capsular types or biovars [1, 3]. The functions and target genes of these factors are detailed in Table 1 and include those encoding outer membrane and porin proteins (*oma87, ompH, plpB*, and *psl*), adhesins (*fimA, pflA, ptfA, hsf-1*, and *hsf-2*), superoxide dismutases (*sodA and sodC*), iron-acquisition-related factors (*exxB, exbBD-tonB, fur, tbpA, hgbA*, and *hgbB*), neumurinidas (*nanB and nanH*), hyaluronidase (*pmHAS*), and toxin (*toxA*). Identifying which virulence factors are prevalent is necessary to predict the pathogenic behaviour of the isolates and select potential future vaccine candidates.

Antimicrobial resistance in pathogenic bacteria from food animals and the environment has become a global public health issue. Although beta-lactams, trimethoprim, colistin, fosfomycin, and macrolides, and tetracyclines have been shown to be the best antimicrobials for treating PRDC [6], resistance to these antimicrobials has been detected previously in *P. multocida* in many countries [3, 22–24]. In Korea, *P. multocida* isolates from pigs are sensitive to most antimicrobials with other respiratory pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV; 61.3%), porcine circovirus type 2 (PCV2; 37.5%), or *Streptococcus suis* (S. suis; 20.0%). *Mycoplasma hyorhinis* (MHR), *Actinobacillus pleuropneumoniae* (APP), *Mycoplasma hyopneumoniae* (MHP), *Haemophilus parasuis* (HPS), *Trueperella pyogenes (T: pyogenes)*, and swine influenza virus (SIV) were detected to a lesser extent (19.2, 14.2, 10.4, 10.0, 4.6, and 3.8%, respectively). Of the *P. multocida* isolates, 166 were included in this study.

**Results**

**Prevalence of *P. multocida* in porcine pneumatic lungs**

In total, 240 *P. multocida* isolates (16.8%) were recovered (Table 2); *P. multocida* was the second most frequently isolated bacterial pathogen in this study. Most isolates (85.0%; 204/240) were detected simultaneously with other respiratory pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV; 61.3%), porcine circovirus type 2 (PCV2; 37.5%), or *Streptococcus suis* (S. suis; 20.0%). *Mycoplasma hyorhinis* (MHR), *Actinobacillus pleuropneumoniae* (APP), *Mycoplasma hyopneumoniae* (MHP), *Haemophilus parasuis* (HPS), *Trueperella pyogenes* (T: pyogenes), and swine influenza virus (SIV) were detected to a lesser extent (19.2, 14.2, 10.4, 10.0, 4.6, and 3.8%, respectively). Of the *P. multocida* isolates, 166 were included in this study.

**Subspecies, biovar, and capsular type determination**

The distribution of biovars and capsular types among the studied *P. multocida* isolates is shown in Table 3. All 166 isolates were identified as *P. multocida* subspecies *multocida*, which produces acid from sorbitol and glucose but not from dulcitol, lactose, and maltose. Most ODC-producing isolates belonged to biovar 3 (68.7%), followed by biovars 2 (21.1%) and 1 (1.8%). Interestingly, 14 isolates (8.4%) displayed identical carbohydrate fermentation results to biovar 3, except for ODC activity, and were thus assigned to biovar 13. All biovar 1 and 2 isolates comprised capsular type F and A, respectively (*P < 0.001*), whereas biovar 3 isolates comprised capsular types A and D (*P < 0.001*), and biovar 13 comprised capsular types A and D (*P > 0.05*). Capsular type A (69.9%) isolates were the most prevalent, followed by types D (28.3%) and F (1.8%), with none of the isolates in this study identified as type B or E. Importantly, this is the first report of capsular type F/biovar 1 isolation since 2014 (Table 3).

**Distribution of virulence-associated genes**

Results of polymerase chain reaction (PCR) analysis of 21 virulence-associated genes showed that all *P. multocida* isolates harboured 14 genes (*oma87, ompH, plpB, psl, fimA, hsf-2, sodA, sodC, exbB, ExbBD-tonB, fur,

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**Table 2** Prevalence of respiratory pathogens and the frequency of *Pasteurella multocida* co-infection with other pathogens

| Bacteria* | PM | SS | MHR | APP | HPS | MHP | TP | PRRSV | PCV2 | SIV |
|-----------|----|----|-----|-----|-----|-----|----|--------|------|-----|
| Total no. (%) of samples in which pathogens were detected | 1430 | 240 | 251 | 199 | 130 | 110 | 96 | 6.7 | 47 | 715 | 456 | 49 | 323 |
| (16.8) | (17.6) | (13.9) | (9.1) | (7.7) | (3.3) | | | | | (50.0) | (31.9) | (3.4) | (22.6) |
| Total no. (%) of sample co-infected with *P. multocida* | 240 | 240 | 48 | 46 | 24 | 25 | 11 | 2.3 | 2.8 | 147 | 90 | 37.5 | 9 | 3.8 |
| (100) | | (20.0) | (19.2) | | | | | | | (61.3) |

*PM Pasteurella multocida, SS Streptococcus suis, MHR Mycoplasma hyorhinis, APP Actinobacillus pleuropneumoniae, HPS Haemophilus parasuis, MHP Mycoplasma hyopneumoniae, TP Trueperella pyogenes
PRRSV porcine reproductive and respiratory syndrome virus, PCV2 porcine circovirus type 2, SIV swine influenza virus
None, none of the respiratory pathogen was detected from pneumonic lungs
**Table 3** Distribution of biovars and capsular types among *P. multocida* isolates from 2008 to 2016

| Biovar  | Capsular type | Total No. (%) | No. (%) of positive isolates within the following years |
|---------|---------------|---------------|-------------------------------------------------------|
|         |               | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 |
| Biovar 1 | Cap F | 3 (1.8) *** | 0 | 0 | 0 | 0 | 0 | 2 (14.3) | 1 (4.0) | 0 |
| Biovar 2 | Cap A | 35 (21.1) *** | 1 (10.0) | 0 | 8 (34.8) | 2 (18.2) | 4 (23.5) | 8 (25.8) | 3 (21.4) | 5 (20.0) | 4 (13.3) |
| Biovar 3 | Cap A | 68 (41.0) *** | 6 (60.0) | 2 (40.0) | 8 (34.8) | 2 (18.2) | 3 (17.6) | 14 (45.2) | 6 (42.9) | 15 (60.0) | 12 (40.0) |
| Biovar 3 | Cap D | 46 (27.7) *** | 3 (30.0) | 2 (40.0) | 4 (17.4) | 4 (36.4) | 7 (41.2) | 9 (29.0) | 3 (21.4) | 3 (12.0) | 11 (36.7) |
| Biovar 13 | Cap A | 13 (7.8) | 0 | 0 | 3 (13.0) | 3 (27.3) | 3 (17.6) | 0 | 0 | 1 (4.0) | 3 (10.0) |
| Biovar 13 | Cap D | 1 (0.6) | 0 | 1 (20.0) | 0 | 0 | 0 | 0 | 0 | 0 |
| Total    |       | 166 | 10 | 5 | 23 | 11 | 17 | 31 | 14 | 25 | 30 |

**Table 4** Distribution of virulence-associated (VA) genes according to capsular type and biovar in 166 *P. multocida* isolates

| VA genes | Type A (%) | Type D (%) | Type F (%) | Biovar 1 (%) | Biovar 2 (%) | Biovar 3 (%) | Biovar 13 (%) | Total No. (%) |
|----------|------------|------------|------------|-------------|-------------|-------------|---------------|---------------|
| **toxA** | 8 (6.9)    | 1 (2.1)    | 0          | 0           | 0           | 9 (7.9) *    | 0             | 9 (5.4)       |
| **pfhA** | 35 (30.2) *** | 0          | 3 (100) ** | 3 (100) **  | 35 (100) *** | 0           | 0             | 38 (22.9)     |
| **hgbB** | 81 (69.8) *** | 46 (97.9) *** | 3 (100) *  | 3 (100) 0    | 113 (99.1) ***  | 14 (100) *  | 130 (78.3)    |               |
| **ptfA** | 116 (100) *** | 46 (97.9) 3 (100) | 3 (100) | 3 (100) | 35 (100) | 113 (99.1) | 14 (100) | 165 (99.4) |

*P < 0.05, **P < 0.01, ***P < 0.001

**Antimicrobial susceptibility**

The antimicrobial-resistance patterns, cumulative minimum inhibitory concentrations (MICs), MIC\textsubscript{50}, and MIC\textsubscript{90} of *P. multocida* isolates from diseased pigs are shown in Table 6. Of the 18 antimicrobials tested, isolates exhibited the highest level of resistance to oxytetracycline (63.3%), followed by florfenicol (16.3%), penicillin (9.0%), ampicillin (7.8%), trimethoprim-sulfamethoxazole (3.0%), enrofloxacin (2.4%), and tulathromycin (0.6%), whereas all isolates were susceptible to ceftriaxone and tilmicosin. The MIC\textsubscript{90} values of antimicrobials for which breakpoints had not been determined according to Clinical and Laboratory Standards Institute (CLSI) criteria were as follows: chlortetracycline (2 μg/mL), spectinomycin (32 μg/mL), clindamycin (16 μg/mL), danofloxacin (0.5 μg/mL), gentamicin (4 μg/mL), neomycin (16 μg/mL), sulfadimethoxine (≥512 μg/mL), tiamulin (32 μg/mL), and tylosin (32 μg/mL).

**Discussion**

Our findings showed that *P. multocida* isolates were prevalent (16.8%) in pig farms and the second most frequently isolated bacterial pathogen from diseased pigs, following *S. suis* (17.6%). This was consistent with previous studies in Korea that reported the prevalence of *P. multocida*. Moreover, our findings demonstrated a high level of antimicrobial resistance among *P. multocida* isolates. The most resistant antimicrobial was oxytetracycline, with a resistance rate of 63.3%. This is consistent with previous studies in Korea, which reported a high level of resistance to oxytetracycline among *P. multocida* isolates. The resistance rate was higher than that reported in other studies, which may be due to the widespread use of oxytetracycline in pig farms in South Korea.

In conclusion, *P. multocida* is a prevalent and important pathogen in pig farms in South Korea, and antimicrobial resistance is a significant concern. Further studies are needed to investigate the factors contributing to antimicrobial resistance and to develop effective strategies to control *P. multocida* infections.
multocida to be between 10 and 15.6% [7, 8]. The infections in this study comprised of a mix of P. multocida (85.0%) with other respiratory pathogens, particularly PRRSV (61.3%; \(P = 0.0001\)). Therefore, veterinary practitioners and surveillance stakeholders should consider coinfection with various pathogens that might exist in a given herd for PRDC control.

We characterised 166 P. multocida isolates by determining their subspecies, biovar, capsular type, virulence-associated genes, and MIC. To the best of our knowledge, this is the first report of biovar prevalence in Korea. All isolates belonged to subspecies multocida, and the most prevalent type was biovar 3 (68.7%), which is consistent with the results of previous studies of P. multocida recovered from pigs [1, 10, 13]. P. multocida biovar 1 is frequently isolated from poultry, but not pigs [1]. We found that the prevalence of biovar 13 was 8.4%, which is slightly higher than that in other countries, such as Australia (2.0%) and Hungary (4.8%) [10, 11]. In agreement with numerous previous studies, the dominant P. multocida capsular types recovered from pneumonic pig lungs were capsular types A (69.9%) and D (28.3%) [1, 15, 16, 26]. Additionally, capsular type B is the etiological cause of septicaemic pasteurellosis, whereas type F is rarely reported in pigs [1, 14]. Interestingly, capsular type F has been isolated in Korea post 2014, although at relatively low proportions \((n = 3; 1.8\%)\), the prevalence of which is consistent with that reported in other European studies \([0.3\% (Germany), 1.0\% (UK), and 2.4\% (Spain)]\) [1, 2, 16]. A recent Chinese experimental study indicated that pig-origin capsular type F isolates are associated with porcine pneumonia and exhibit high pathogenicity in pigs [27]. Additionally, we found that P. multocida capsular type F was the only relevant respiratory pathogen isolated from three growing pigs with moderate-to-severe suppurative bronchopneumonia with fibrous/fibrinous

### Table 5 Distribution of the toxA, hgbB, and pfhA gene profiles according to biovars

| Gene profile of toxA/hgbB/pfhA | Biovar 1 (n = 3) | Biovar 2 (n = 35) | Biovar 3 (n = 114) | Biovar 13 (n = 14) | Total (n = 166) |
|-------------------------------|----------------|-----------------|------------------|------------------|----------------|
| toxA−hgbB−pfhA− | 3 (100) | 0 | 0 | 0 | 3 |
| toxA−hgbB−pfhA− | 0 | 35 (100)*** | 0 | 0 | 35 |
| toxA−hgbB−pfhA− | 0 | 0 | 9 (7.9)† | 0 | 9 |
| toxA−hgbB−pfhA− | 0 | 0 | 104 (91.2)*** | 14 (100)† | 118 |
| toxA−hgbB−pfhA− | 0 | 0 | 1 (0.9) | 0 | 1 |

†\(P < 0.05\), ***\(P < 0.001\)

### Table 6 Antimicrobial susceptibility and cumulative percentage of P. multocida isolates (n = 166) for 18 antimicrobials

| Antimicrobial | Cumulative percentage of strains inhibited as antimicrobial concentration (\(\mu\)g/mL) of | MIC\textsubscript{50} (\(\mu\)g/mL)\textsuperscript{a} | MIC\textsubscript{90} (\(\mu\)g/mL)\textsuperscript{a} | S (%)\textsuperscript{b} | I (%)\textsuperscript{b} | R (%)\textsuperscript{b} | MIC Breakpoint (\(\mu\)g/mL)\textsuperscript{c} |
|---------------|---------------------------------|------------------|------------------|----------------|----------------|----------------|------------------|
| Oxytetracycline | 25.3 | 2 | 16 | 25.3 | 11.5 | 65.3 | 32 |
| Fluraxinol | 25.3 | 0.5 | 8 | 82.5 | 11.3 | 5.2 | 16 |
| Pencillin | 69.3 | 0.12 | 0.25 | 90.4 | 0.9 | 9 | 1 |
| Ampicillin | 89.8 | 0.25 | 0.5 | 91.8 | 0.7 | 7.8 | 2 |
| Trimethoprim/sulfamethoxazole | 97 | 2 | 2 | 94.4 | 4.3 | 7.4 | 2 |
| Streptomycin | 87.3 | 0.12 | 0.25 | 93.4 | 4.3 | 7.4 | 2 |
| Tetracycline | 81.9 | 0.1 | 0 | 94.4 | 0.6 | 6 | 64 |
| Cefoxitin | 99.4 | 0.1 | 2 | 94.4 | 0.6 | 6 | 64 |
| Tetracycline | 75.9 | 0.25 | 100 | 0 | 0 | 0 | 0 |
| Chloramphenicol | 18.7 | 0.16 | 1 | 32 | 0 | 6 | 100 |
| Spectinomycin | 8.4 | 0.01 | 0 | 32 | 0 | 6 | 100 |
| Cloxacillin | 97.8 | 0.1 | 0 | 32 | 0 | 6 | 100 |
| Dicloxacillin | 84.3 | 0.12 | 0.5 | 32 | 0 | 6 | 100 |
| Gentamicin | 10.2 | 0.16 | 1 | 32 | 0 | 6 | 100 |
| Neomycin | 57.8 | 0.04 | 16 | 32 | 0 | 6 | 100 |
| Sulbactam | 48.8 | 0.512 | 0.512 | 0 | 0 | 0 | 0 |
| Tetracycline | 3 | 3 | 36.1 | 100 | 0 | 0 | 0 |
| Tylosin | 5.6 | 3 | 3 | 50.8 | 0 | 0 | 0 |

The grey zone represents the tested concentration range of each antimicrobial provided in the BOPO6F plate. Susceptibility and resistance breakpoints are indicated by double vertical (sensitive) and single vertical (resistant) lines according to the guidelines of each reference. MIC\textsubscript{50} and MIC\textsubscript{90} concentrations at which the growth of 50 and 90%, respectively, of the isolates is inhibited.

\(\textsuperscript{a} \text{MIC}\textsubscript{50} and \text{MIC}\textsubscript{90} \text{ breakpoint were those recommended by the Clinical and Laboratory Standards Institute (CLSI); trimethoprim/sulfamethoxazole interpretation was based on a previous study}\) [25].
pleuritis. This represents the first report identifying capsular type F isolates in Korea; therefore, the pathogenic significance of type F in pigs needs to be elucidated.

Virulence genotyping is a useful typing method for molecular characterisation of bacterial pathogens and has been previously applied to *P. multocida* [1, 3]. Although *omaB*, *ompH*, *plpB*, *psl*, *fimA*, *hsf-2*, *sodA*, *sodC*, *exbB*, *ExbBD-tonB*, *fur*, *hgbA*, *nanB*, *nanH*, and *ptfA* were uniformly distributed among the isolates tested, none possessed *tbpA*, which agreed with the results of previous pig studies [1–3, 17]. The wide distribution of these genes indicates their importance for the survival of *P. multocida* within the host environment. Additionally, the virulence factors involved in cross-protection might constitute potential vaccine candidates, regardless of capsular type [3].

However, previous studies demonstrated that several non-uniformly distributed virulence-associated genes exhibit significant relatedness with specific capsular types [1, 3, 8, 17]. As shown in Table 4, all capsular type A and D isolates harboured *pmHAS* and *hsf-1*, respectively, and most type D isolates harboured *hgbB* (P < 0.001). In this study, capsular type F displayed virulence-associated gene profiles similar to those of capsular type D (*hsf-1‘hgbB’*), except for *pfhA*. Previous studies reported *toxA* as clearly associated with type D [1, 3, 7, 17]; however, we found that only one of the 47 type D (2.1%) isolates and 6.9% of type A isolates harboured *toxA*. These results, however, are not significant because most of the isolates were from pneumonic lesions and not from turbinates with PAR. Similar to a previous report, distinct associations were observed between the virulence-associated gene profiles of *toxA*, *hgbB*, and *pfhA* and biovars, except for biovar 13 [1]. All biovar 1, 2, and 13 isolates exhibited *toxA* *hgbB* *pfhA* (P < 0.001), *toxA* *hgbB* *pfhA* (P < 0.001), and *toxA* *hgbB* *pfhA* (P < 0.005) profiles, respectively, and most biovar 3 isolates displayed a *toxA* *hgbB* *pfhA* profile (P < 0.001). Additionally, *toxA* was found only in biovar 3 isolates (*toxA* *hgbB* *pfhA*; P < 0.05).

Swine diseases have become co-infected with immunosuppressive diseases, leading to antimicrobial treatment failure and frequent resistance occurrence. Treatment against *P. multocida* infections commonly includes broad-spectrum antimicrobials [3]. In this study, beta-lactams (penicillin, ampicillin, and ceftiofur), macrolides (tulathromycin and tilmicosin), and fluoroquinolone (enrofloxacin) were found to be more effective than oxytetracycline and florfenicol. Therefore, these agents are recommended as empirical antimicrobials for the treatment of *P. multocida* infection. Tetracycline resistance has previously been reported in *P. multocida* isolates worldwide [3, 6, 25, 28, 29]. Its prevalence in the present study was found to be 63.3%, which is similar to the prevalence in China (58.0%) and North America (53.4%) [3, 28] but higher than that in Australia (28.0%) and European countries (20.4%).

Previous studies recommended the use of florfenicol for the treatment of infections caused by *P. multocida*, because florfenicol-resistance rates are very low (0–2%) in China, North America, Australia, and Europe [6, 25]; however, the present study showed a relatively higher resistance (16.3%). According to the Korea Animal Health Products Association, tetracyclines and florfenicol are the most commonly used antibiotics in Korean pig husbandry [30], with their frequent use reflected in the resistance rates in the present study. Based on the occurrence of high rates of tetracycline and florfenicol resistance, these antimicrobial agents should be used carefully and accompanied by susceptibility tests. Additionally, continuous surveillance of antimicrobial resistance in respiratory pathogens, including *P. multocida*, is required due to the increasing use of therapeutic antimicrobials and emergence of new resistant strains.

This study was conducted to determine the phenotypic and genotypic characteristics of swine *P. multocida* isolates in Korea. However, the collected samples cannot be representative of current *P. multocida* isolates in Korean swine farms, given that the number of isolates submitted annually varies, and the isolates used in this study originated from diagnostic samples with unknown antimicrobial-treatment history. However, a large-scale study for the characterisation of clinical lung samples of *P. multocida* isolates would sufficiently broaden the understanding of *P. multocida* as a respiratory pathogen.

**Conclusions**

This represents a comprehensive report of *P. multocida* isolates in pigs in Korea. Our findings provide scientific information for further research, including development of vaccine candidates and guidelines for antimicrobial use in veterinary medicine. Moreover, the low discriminatory power of phenotypic characterisation limits the scope of adequate epidemiological information; therefore, different genotyping techniques using pulsed-field gel electrophoresis or multilocus sequence typing might be required to further elucidate the epidemiology of *P. multocida* and its genetic relatedness.

**Methods**

**Bacterial isolation and identification**

In total, 1430 lung samples were collected from pigs (suckling pigs, 9%; weaning pigs, 49%; growing-finishing pigs, 23%; and unknown, 19%) with pneumonic gross lesions from 514 farms nationwide between 2008 and 2016. All lung samples were submitted to the Animal and Plant Quarantine Agency for differential diagnosis of porcine respiratory diseases, including APP, HPS, *S. suis*, *T. pyogenes*, MHP, MHR, PRRSV, PCV2, and SIV. Following gross and histopathologic examination,
samples were cultured on 5% sheep blood agar, chocolate agar (Asan Pharm. Co., Ltd., Seoul, Korea), and MacConkey agar (Becton Dickinson, Sparks, MD, USA) and then incubated aerobically at 37°C for 48 h. Suspected mucoid and non-haemolytic colonies were subjected to Gram staining and biochemical identification using the VITEK II system (BioMérieux, Marcy l’Etoile, France). Identification was further confirmed by species-specific PCR assay for amplification of knt1 (Table 1) [19]. All P. multocida isolates were stored at −80°C until use to determine the subspecies, biovar, and capsular type. Previously reported methods were used to differentiate between P. multocida and other pathogens [3, 31, 32].

Subspecies and biovar determination
The confirmed P. multocida isolates were classified into three subspecies (multocida, septica, and gallicida) based on sorbitol and dulcitol fermentation [9]. Additionally, isolates were assigned to one of the established biovars based on their ability to ferment carbohydrates (sorbitol, dulcitol, maltose, xylose, glucose, trehalose, lactose, and arabinose) and produce the ODC enzyme [10].

PCR assay for capsular typing and virulence-associated gene detection
P. multocida isolates were inoculated into brain-heart infusion broth (Becton Dickinson) and cultured for 18 h. Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. The capsular types of the isolates were determined by multiplex PCR using the capsule-specific primers shown in Table 1 [20]. PCR analysis of 21 virulence-associated genes, including oma87, ompH, pIpB, psL, fimA, pflA, pflA, hisF-1, hisF-2, sodA, sodC, ebxB, ebxBD-tonB, fur, tbaA, hgbA, hgbB, nanB, nanH, pmH1AS, and toxA (Table 1) [3, 17, 18, 21], was conducted as previously described. PCR amplification was performed using a Mastercycler ep Gradient S (Eppendorf, Hamburg, Germany), and amplified products were analysed with a capillary electrophoresis system (QIAxcel Advanced System; Qiagen). All tests were performed in duplicate in parallel with the relevant positive and negative controls.

Antimicrobial-susceptibility testing
The MIC of all isolates (n = 166) was determined using the standard broth microdilution method with the Sensititre system (TREK Diagnostic System; Thermo Fisher Scientific, Cleveland, OH, USA) and commercially prepared 96-well antimicrobial testing plates containing 18 different agents (BOPO6F; TREK Diagnostic Systems). The following antimicrobials were tested: penicillin, ampicillin, ceftiofur, florfenicol, gentamicin, neomycin, chlorotetracycline, oxytetracycline, clindamycin, enrofloxacin, danofloxacin, trimethoprim/sulfamethoxazole, sulfadimethoxine, spectinomycin, tulathromycin, tylosin, tetracycline, tilmicosin, and tiamulin. Escherichia coli ATCC 25922 was tested for quality control purposes. As shown in Table 6, the MICs were interpreted according to CLSI guidelines for oxytetracycline, florfenicol, penicillin, ampicillin, enrofloxacin, tulathromycin, ceftiofur, and tilmicosin or those of a previous study describing analysis of trimethoprim/sulfamethoxazole, for which CLSI breakpoints were not available [25, 33]. The overall MIC50 and MIC90 values (i.e., the lowest concentrations at which growth was inhibited by 50 and 90%, respectively) for each antimicrobial were determined for all isolates.

Statistical analysis
Statistical testing was performed using GraphPad Prism (v5.01; GraphPad Software, San Diego, CA, USA) and SPSS (v22.0; IBM Corp., Armonk, NY, USA). Pearson’s chi-squared and Fisher’s exact tests were used to assess associations among capsular types, biovars, and virulence-associated genes. A P < 0.05 was considered statistically significant.

Abbreviations
APP: Actinobacillus pleuropneumoniae; CLSI: Clinical and Laboratory Standards Institute; HPS: Haemophilus parasuis; MHP: Mycoplasma hyopneumoniae; MHR: Mycoplasma hyorhinis; MIC: Minimum inhibitory concentration; ODC: Ornithine decarboxylase; P. multocida: Pasteurella multocida; PAR: Progressive atrophic rhinitis; PCR: Polymerase chain reaction; PCV2: Porcine circovirus type 2; PRDC: Porcine respiratory disease complex; PRRSV: Porcine reproductive and respiratory syndrome virus; S. suis: Streptococcus suis; SIV: Swine influenza virus; T. pyogenes: Trueperella pyogenes

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Availability of data and materials
The datasets generated or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
JK and SIO were involved in study design, performed sample collection, conducted laboratory work, and drafted the manuscript. JWK helped design the study and interpret the data. BS and WIK were involved in designing and conducting laboratory work, and drafted the manuscript. All authors reviewed the article and approved it.

Ethics approval and consent to participate
Non-experimental study has been performed on animals. Diagnostic investigation and additional characterisation have been conducted on samples that are submitted by veterinarians and pig owners. The specimens used in this research are field samples originating from natural respiratory symptoms and sent to our laboratory for official diagnosis.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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