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Serovar and multilocus sequence typing analysis of *Pasteurella multocida* from diseased pigs in Taiwan

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Abstract

Background *Pasteurella multocida* causes progressive atrophic rhinitis and suppurative bronchopneumonia in pigs, which results in severe economic losses in swine industry. This study aimed to determine the serovar, genotype and prevalence of *toxA* virulence gene of *Pasteurella multocida* isolates collected in Taiwan. A total of 164 *Pasteurella multocida* isolates from 161 diseased pigs were characterized by serotyping, multilocus sequence typing (MLST), antimicrobial susceptibility testing, and the presence of virulence gene (*toxA*) and antibiotic resistance gene (*floR*).

Results The majority of *Pasteurella multocida* strains were serovar D:L6 (48.2%; 79/164) followed by A:L6 (28.7%; 47/164) and A:L3 (19.5%; 32/164). More than 80% of strains carrying *toxA* gene belonged to serovar A:L6 (82.6%; 19/23). The MLST data showed five sequence types (STs), where multi-host ST10 was the most dominant. Most *Pasteurella multocida* strains of multi-host ST10 were serovar A:L6 (93.9%; 31/33), which suggested that STs were highly associated with specific serovars. Most of the *floR*-carrying *Pasteurella multocida* strains belonged to serovar D:L6 with significantly high resistance to some antimicrobial agents, especially florfenicol.

Conclusions This study demonstrated that serovar D:L6 and multi-host ST10 was the most prevalent *Pasteurella multocida* strain in Taiwan. A:L6 accounted for the majority of *tox*A-positive strains and the presence of *floR* gene may be responsible for the antimicrobial resistance to florfenicol.

Keywords *Pasteurella multocida*, Molecular serotyping, Multilocus sequence typing, Antimicrobial susceptibility testing, *floR* gene

Background

Pasteurella multocida, which belongs to the family *Pasteurellaceae*, infects a wide range of hosts including pigs, chicken, cattle, sheep, rabbits, and humans. Different hosts show different symptoms after infection [1]. *Pasteurella multocida* is an important opportunistic pathogen in swine, causing suppurative bronchopneumonia and progressive atrophic rhinitis (PAR), and is responsible for significant losses worldwide [1–3]. Prevalence of *Pasteurella multocida*has has been reported as 16.8% in diseased pigs with pneumonia in Korea [4], and as 8% in pigs with pneumonia or PAR in China [5]. Capsules and lipopolysaccharides (LPS) are two types of antigens used for serotyping of *Pasteurella multocida*. Based on antigenic differences, passive hemagglutination tests are used to classify *Pasteurella multocida* capsule antigens into 5 serovars (A, B, D, E, and F) [6–8]. According to the results from Heddleston et al. using gel diffusion immunoprecipitation test, the serovars of *Pasteurella multocida* LPS can be determined as Heddleston

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serovar 1 (H1)-H16 [9–11]. To date, multiplex polymerase chain reaction (mPCR) is widely used to identify capsule and LPS serovars of *Pasteurella multocida*. As passive hemagglutination test, mPCR is used to classified capsule antigens into 5 serovars, but some strains are yet to be identified due to the loss of capsules [12]. Additionally, Harper et al. developed LPS-mPCR according to the genetic organization of the LPS outer core biosynthesis loci, which identified *Pasteurella multocida* strains into serovars L1-L8 to replace Heddleston serotyping scheme (H1-H16) [13].

Pasteurella multocida toxin (PMT), a dermonecrotic toxin, is an important virulence factor of PAR in the swine. PMT inhibits osteoblastic differentiation and stimulates the differentiation of osteoclasts, which leads to atrophy and defect of the diaphragm in the snout [14, 15]. Encoded by *toxA* gene, PMT is produced by capsular type D and some type A *Pasteurella multocida* strains [14].

Florfenicol is a broad spectrum bacteriostatic agent. Florfenicol can be administered via either intramuscular (*i.m.*) route or orally in water or feed, and it is widely used for treating swine respiratory infections such as *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Glaesserella parasuis* and *Streptococcus suis* [16–18]. However, several florfenicol resistance genes have been reported in *Pasteurella multocida*. Among them, *floR* is critical for the resistance of florfenicol. The *floR* gene is mainly located in certain plasmids (e.g., pCCK381 and pCCK1900), which play an important role in the horizontal transfer of resistance genes, not only between different *Pasteurella multocida* strains but also between *Pasteurella multocida* and other bacteria [19].

Genetic diversity of bacteria is associated with pathogenicity [20]. Using multi-locus sequence typing (MLST), we can amplify housekeeping gene loci and define genetic diversity by allocating bacterial strains to sequence types (STs) [21]. Once we determine the STs, we can further analyze any differences in pathogenicity linked genes. There are two MLST databases regarding *Pasteurella multocida* on the PubMLST website (https://pubmlst.org/), i.e., the Rural Industries Research and Development Corporation (RIRDC) and the multiple host (multihost) MLST databases. Up to January of 2024, 452 and 209 STs have been documented in the RIRDC and multihost MLST databases, respectively.

In this study, we characterized *Pasteurella multocida* isolates from diseased swine in Taiwan by analysis of serovars, antimicrobial susceptibility testing, presence of virulence and florfenicol-resistance gene, and MLST for epidemiological comparison.

Results

Serovars, LPS genotypes and the *toxA* gene

We collected 164 *Pasteurella multocida* samples, in which 146 samples (89%) were isolated from the lung or bronchi and 18 samples (11%) were isolated from the nostril or turbinate. In these strains, two capsule serovars (A and D) and two LPS serovars (L3 and L6) were detected. The serovar percentages of the lung or bronchi strains in decreasing order were D:L6 (45.9%; 67/146), A:L6 (28.8%; 42/146), A:L3 (21.2%; 31/146), D:L3 (1.4%; 2/146), UN:L3 (1.4%; 2/146), and UN:L6 (1.4%; 2/146). Four strains (2.7%; 4/146) from the lung or bronchi were non-typeable in this study. The serovar percentages of the nostril or turbinate strains were D:L6 (66.7%; 12/18), A:L6 (27.8%; 5/18) and A:L3 (5.6%; 1/18). Taken together, the dominant serovar of isolated strains from respiratory system was D:L6 (Table 1).

Twenty-three (14.0%; 23/164) *Pasteurella multocida* isolates, including 20 (87.0%; 20/23) lung or bronchi isolates and 3 (13.0%; 3/23) nostril or turbinate isolates, had *toxA* gene. In addition, 19 *Pasteurella multocida* strains (82.6%; 19/23) with *toxA* gene were serovar A:L6, followed by serovars D:L6 (8.7%; 2/23) and UN:L6 (8.7%; 2/23) (Table 2). Notably, there were two herds, each containing multiple strains from different sites of isolation in a diseased pig. We observed that the two isolates collected from a diseased pig in one of the two infected herds showed the same serovar (A:L6) with different presence of *toxA* gene. However, the three isolates from

Table 1 Distribution of isolation sites and serovars among *Pasteurella multocida* isolates ($n = 164$)

Isolation sites	Number of isolates with serovars[a]						Total
	A: L3	A: L6	D: L3	D: L6	UN[b]: L3	UN[b]: L6	
Lung/ bronchi	31	42	2	67	2	2	146 (89.0%)
Nostril/ turbinate	1	5	-	12	-	-	18 (11.0%)
Total	32 (19.5%)	47 (28.7%)	2 (1.1%)	79 (48.2%)	2 (1.2%)	2 (1.1%)	164 (100.0%)

[a] Serovars include capsule serovar and LPS serovar

[b] UN: non-typeable

Table 2 Prevalence of virulence and antibiotic resistance genes in *Pasteurella multocida* isolates ($n = 164$) and the relationships to capsular and LPS serovars

Genes	Serovars						Total
	A: L3	A: L6	D: L3	D: L6	UN[a]: L3	UN[a]: L6	
toxA	-	19	-	2	-	2	23 (14.0%)
floR	11	15	2	43	1	-	72 (43.9%)

[a] UN: non-typeable

the other infected herd showed different serovars (A:L6, D:L6, UN:L6) and presence of *toxA* gene.

Antimicrobial susceptibility testing

The MICs of the 164 *Pasteurella multocida* isolates are shown in Table 3. The results demonstrated the highest susceptibility to ceftiofur (99.4%), and high susceptibility to enrofloxacin, cefazolin, flumequine, and ampicillin. The proportion of *Pasteurella multocida* susceptible to these antimicrobial agents was higher than 70%. The isolates showed moderate susceptibility (40–70%) to doxycycline, erythromycin, kanamycin, florfenicol, and lincospectin; and low susceptibility to trimethoprim/sulfamethoxazole, tiamulin, lincomycin, and tylosin, in which the proportion of *Pasteurella multocida* susceptibility was lower than 40%. The *Pasteurella multocida* isolates showed the highest resistance to tylosin (98.8%).

There were 160 (97.6%; 160/164) *Pasteurella multocida* isolates resistant to three or more classes of antimicrobial agents, and they were considered as multi-drug resistant isolates. Among these multi-drug resistant isolates, 40 isolates (25%; 40/160) that were resistant to five classes of antimicrobial agents represented the most common antimicrobial resistant pattern. In addition, there were six isolates resistant to nine classes of antimicrobial agents (Table S1).

There were 72 (43.9%; 72/164) strains containing the *floR* gene. Among them, 62 strains (86.1%) were isolated from the lung or bronchi and 10 strains (13.9%) were isolated from the nostril or turbinate. According to the serotyping data, 43 strains (59.7%; 43/72) with the *floR* gene belonged to serovar D:L6, followed by A:L6 (20.8%; 15/72), A:L3 (15.3%; 11/72), D:L3 (2.8%; 2/72), and UN:L3 (1.4%; 1/72) (Table 2). Most of the 72 strains (93.1%; 67/72) with *floR* gene were resistant to florfenicol and these strains were multi-drug resistant. Notably, there were five herds, each containing two strains from two diseased pigs. We observed that the two strains from

Table 3 Antimicrobial susceptibility profiles and resistant percentage of *Pasteurella multocida*

Antimicrobial agents	Number of MIC values (μg/mL)																MIC_{50}[a] (μg/mL)	MIC_{90}[a] (μg/mL)	R[b] (%)
	>1024	1024	512	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	<0.125			
Ampicillin		17	4	3	9	3	6				2	3	63	54			0.5	1024	26.8
Cefazolin					1	2	6	3	6	4	27	70	23	22			1	8	5.5
Ceftiofur							1					12	19	47		85	<0.125	0.5	0.6
Kanamycin		64	1			1	11	38	42	7							16	1024	40.2
Doxycycline								1	20	33	42	39	19	10			2	8	58.5
Erythromycin		2	7	5		2	4	6	33	57	34	9	5				4	32	36.0
Tylosin	5	13	4	16	31	39	46	8	2								64	1024	98.8
Tiamulin		1		15	37	46	48	16	1								64	128	89.6
Florfenicol			2	3	23	27	17	3	2	3	11	45	28				2	128	47.0
Lincomycin		13		7	38	62	38	5	1								64	256	96.3
Lincospectin		2	1	4	10	34	42	53	18								32	128	56.7
Flumequine				6	2	2	2	5	3	21	40	24	11	34		14	1	16	12.2
Enrofloxacin										1	1	4	8	62		88	<0.125	0.25	3.7
Trimethoprim/sulfamethoxazole		5	19	47	5	4	3	9	21	17	25	6	2	1			32	512	79.3

The dilution ranges tested for each antimicrobial agent are those contained within the white area. Breakpoints are indicated with vertical black lines

[a] MIC_{50} and MIC_{90} are the lowest concentration of antimicrobial agent capable of inhibiting the growth of 50% and 90% of isolates, respectively

[b] The value for R means the percentage of the resistance

each of the five infected herds exhibited different serovars and the presence of the *floR* gene.

Multilocus sequence typing analysis

A total of 70 isolates were randomly selected from the 164 isolates for MLST using the Multi-host scheme. Five STs were generated in this study, which were ST10 (47.1%; 33/70), ST11 (22.9%; 16/70), ST134 (18.6%; 13/70), ST3 (8.6%; 6/70), and ST119 (2.9%; 2/70). There were 61 lung/bronchi isolates and 9 nostril/ turbinate isolates. The 5 STs were distributed over the different tissue sources, with the strains from the lung or nostril being mostly ST10 (Table 4). The ST11 and ST134 strains were serovar D:L6, the ST3 and ST119 strains were serovar A:L3, and most of the ST10 strains were serovar A:L6 (93.9%: 31/33), followed by D:L6 and UN:L6 (Fig. 1). The results indicated that the serovars of the tested strains might be associated with the MLST genotypes (Fig. 2; Table S2).

Nineteen (27.1%; 19/70) among the 70 strains contained the *toxA* gene, in which 1 strain belonged to ST134 and 18 strains belonged to ST10 (94.7%; 18/19). There were 16 strains (84.2%; 16/19) isolated from the lung or bronchi and 3 strains (15.8%; 3/19) from the nostril or turbinate. Sixteen strains from the lung or bronchi and two strains from the nostril or turbinate belonged to ST10, and one strain (33.3%; 1/3) from the nostril or turbinate belonged to ST134 (Fig. 1; Table S3).

Thirty-one of the 70 strains (44.3%; 31/70) contained the *floR* gene. Among them, 24 strains (77.4%; 24/31) were isolated from the lung or bronchi and seven strains (22.6%; 7/31) were isolated from the nostril or turbinate. In addition, 11 strains (35.5%; 11/31) belonged to ST10 and 10 strains (32.3%; 10/31) belonged to ST134, representing the most prevalent STs of the *Pasteurella multocida* strains with *floR* gene (Fig. 1; Table S3).

Co-infection of other respiratory pathogens in the isolates

Pasteurella multocida was isolated with other pathogens in 87.1% (143/164) of the collected samples. Porcine reproductive and respiratory syndrome virus (PRRSV) (68.9%; 113/164), *Mycoplasma hyorhinis* (48.7%; 80/164), *Streptococcus suis* (31.1%; 51/164) and *Glaesserella parasuis* (26.8%; 44/164) were the most frequently isolated pathogens together with *Pasteurella multocida*, followed by porcine circovirus type 2 (PCV2) (15.2%; 25/164), *Actinobacillus pleuropneumoniae* (8.5%; 14/164), *Escherichia coli* (8.5%; 14/164), *Mycoplasma hyopneumoniae* (6.7%; 11/164), *Mycoplasma suis* (3.7%; 6/164) and *Bordetella bronchiseptica* (1.2%; 2/164) (Table 5).

Discussion

The serovar D strains (49.3%; 81/164) were the most common serovar followed by serovar A isolates (48.2%; 79/164) in this study, which is in agreement with the previous studies in China, Korea and Germany [4, 22, 23]. Notably, four strains (2.7%; 4/146) isolated from the lung or bronchi had non-tyepable capsules in this study. Non-typeable strains from the lungs of pigs suffering from pneumonia have been found, indicating that these non-typeable strains may be associated with porcine pneumonia [3, 5, 24]. Sahoo et al. found that the non-typeable strains exhibited the uniform distribution of virulence genes. In addition, the non-typeable serowars showed a higher degree of resistance to penicillins [24]. Although non-typeable *Pasteurella multocida* strains are rare in pigs, those exhibiting antibiotic resistance may pose a pathogenic risk to pigs [3, 24].

Six serovars were detected in this study: D:L6 (48.2%) was the most common serovar, and followed in decreasing order by A:L6 (28.7%), A:L3 (19.5%), UN:L3 (1.2%), D:L3 (1.1%), and UN:L6 (1.1%) (Table 1). The result is similar to a study in China showing D:L6 as the major serovar, and A:L6 (29.6%) and A:L3 (20.0%) as the second most serovars [22]. However, different results were obtained in a study in Hungary that reported A:L3 (72.5%) was the major serovar, and D:L3 (27.5%) was a minor serovar [25]. Notably, the distribution of the different serovars varies both across regions and over time within the same region. The reasons for this tendency are still unclear and further studies are needed for elucidation.

Most of the strains with *toxA* gene were A:L6 (82.6%; 19/23) and a few were D:L6 (8.7%; 2/23), implying that *toxA* gene is highly linked to capsule serovar A. Similar results from Korea and Hungary were reported [4, 25]. Although *toxA* gene is more often found in *Pasteurella multocida* strain with capsular serovar D that causes

Table 4 Distribution of MLST sequence types and isolation sites among *Pasteurella multocida* isolates ($n = 70$)

Isolation sites	Number of isolates with sequence types (STs)					Total (%)
	ST3	ST10	ST11	ST119	ST134	
Lung/ bronchi	6	28	15	2	10	61 (87.1%)
Nostril/ turbinate	-	5	1	-	3	9 (12.9%)
Total (%)	6 (8.6%)	33 (47.1%)	16 (22.9%)	2 (2.9%)	13 (18.6%)	70 (100.0%)

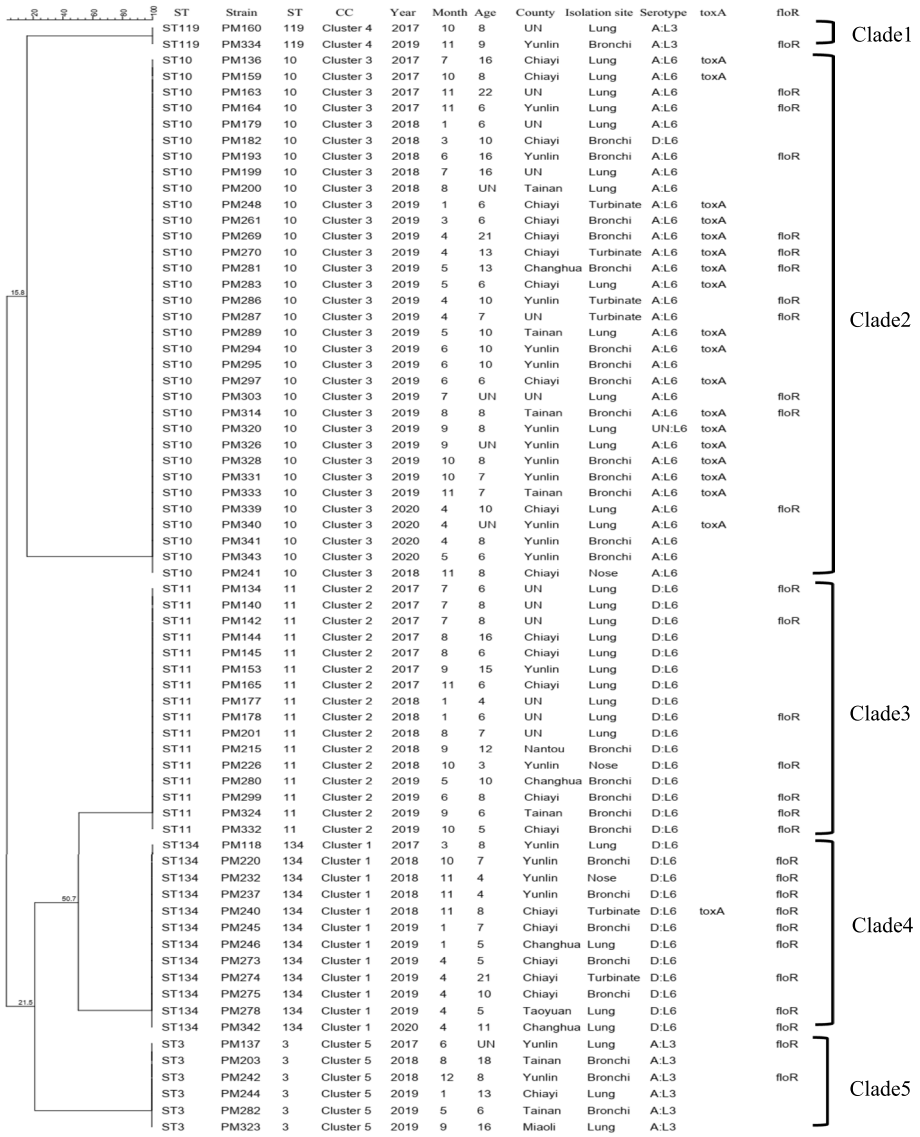

Fig. 1 UPGMA dendrogram constructed from the ST profiles of *Pasteurella multocida* isolates ($n = 70$)

atrophic rhinitis [2, 23, 26, 27], many other studies have reported *toxA* gene detected in *Pasteurella multocida* strains with capsular serovar A from humans, cattle, and sheep [25, 28, 29].

The proportion of isolates showing resistance to florfenicol, and the MIC_{50} and MIC_{90} values of the *Pasteurella multocida* isolates were 47.0%, 2 μg/mL and 128 μg/mL, respectively. The *Pasteurella multocida* strains isolated in Spain and China were susceptible to florfenicol [5, 30], while in Europe and Australia, low-level resistance was observed at 0.7% and 2%, respectively [31, 32]. In addition, the drug resistance is 16.3% in Korea [4]. The MIC_{50} in these reports is mostly 0.5 μg/mL; and the MIC_{90} is no higher than 1 μg/mL except for the study by Kim et al. (8 μg/mL) [4]. Compared with these studies, the drug resistance proportion and MIC value of the *Pasteurella multocida* strains in Taiwan are higher than those in Korea, China, Australia and Europe [4, 5, 31, 32]. The phenomenon that the strains isolated in Taiwan had high drug resistance to florfenicol might have been related to the *floR* gene. There were 43.9% (72/164) of *Pasteurella multocida* strains containing the *floR* gene, and most (93.1%; 67/72) of the strains with florfenicol-resistance gene were multi-drug resistant strains. Strains with the *floR* gene showed a 64-fold increase in MIC_{50} and a two-fold increase in MIC_{90} for florfenicol compared to strains without the gene. Surprisingly, the strains with *floR* gene also had higher MIC values for ampicillin, kanamycin,

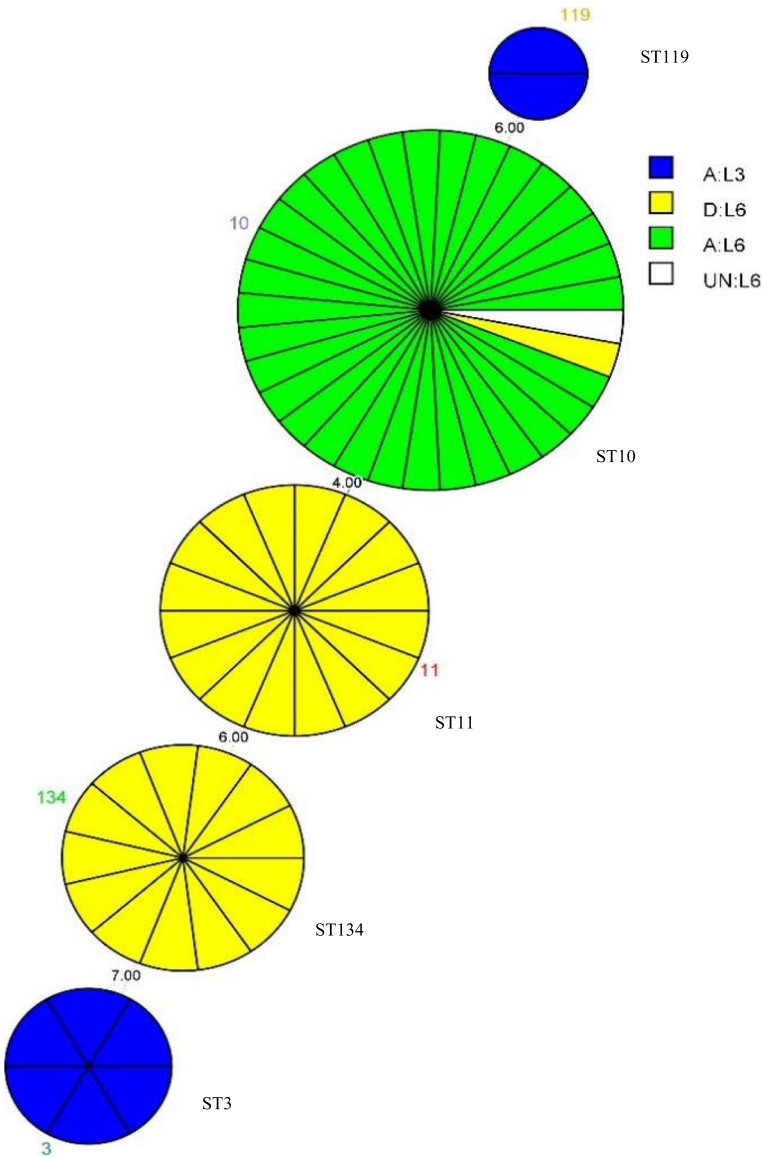

Fig. 2 MST graph analysis of *Pasteurella multocida* isolates ($n = 70$) constructed from the ST profiles. The nodes were labeled as representaive STs that involved the strains with identified serovars, the branch lengths were set according to the discrepancy of allele profile between connected nodes

tiamulin, lincomycin, and tylosin (Figure S1). According to the drug resistance of the prevalent serovars D:L6 and A:L6, there were of no significant difference between cefazolin and flumequine, but D:L6 showed significantly higher drug resistance to most antimicrobial agents in comparison to A:L6 (Figure S2). According to our data, the serovar of the strains with *floR* gene was mostly D:L6 (59.7%; 43/72), whereas A:L6 serovar only accounted for 20.8% of all these drug-resistant strains (Table 2). This result suggested that the D:L6 serovar strains may particularly harbor genes crucial for drug resistance. Several reports have shown that the plasmids carrying *floR* gene often have more than two different drug-resistance genes [19, 33, 34], and this may explain why the *Pasteurella multocida* strains having *floR* gene are resistant not only to florfenicol but also to other antimicrobial agents. The role of plasmids in the drug resistance of these *Pasteurella multocida* strains warrants further investigation.

The ST11 and ST134 strains belonged to D:L6 serovar, the ST3 and ST119 strains belonged to A:L3 serovar, and the ST10 strains mostly belonged to A:L6 serovar, meaning that the STs were associated with certain serovars, similar to many of prior studies [22, 35, 36]. The capsule/LPS/MLST genotype of A/L6/ST10 (44.3%; 31/70)

Table 5 Co-infection of other pathogens with *Pasteurella multocida* ($n=164$)

Type	Pathogen	N	%
Virus	Porcine reproductive and respiratory syndrome virus (PRRSV)	113	68.9
	Porcine circovirus type 2 (PCV2)	25	15.2
Bacteria	*Mycoplasma hyorhinis*	80	48.7
	Mycoplasma hyopneumoniae	11	6.7
	Glaesserella parasuis	44	26.8
	Streptococcus suis	51	31.1
	Mycoplasma suis	6	3.7
	Escherichia coli	14	8.5
	Actinobacillus pleuropneumoniae	14	8.5
	Bordetella bronchiseptica	2	1.2

accounted for the majority of strains in Taiwan, followed by D/L6/ST11 (22.9%; 16/70) (Table S2), whereas D/L6/ST11 (45.0%; 18/40) was the most prevalent in China [22].The results showed that presence of *toxA* gene (94.7%; 18/19) in the *Pasteurella multocida* isolates in Taiwan was significantly associated with ST10 regardless of the isolation site (Table S3). In contrast, Peng et al. found that the *toxA* gene only in ST11 isolates [22]. These results suggested that *toxA* gene exists in different STs, and further studies involving more samples are warranted to determine the association between the *toxA* gene and STs.

Co-infection of bacteria and viruses leads to more severe respiratory diseases than single infection in pig farms [37]. Fablet et al. found that bacteria including *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, and *Glaesserella parasuis* were detected in the lungs of slaughter pigs [38]. In a retrospective study associated with respiratory diseases in pigs in USA, 88.2% of the analyzed cases showed co-infection of two or more pathogens, and PRRSV (35.4% of the samples), *Pasteurella multocida* (31.6%), *Mycoplasma hyopneumoniae* (27%), *Glaesserella parasuis* (22.0%) and PCV2 (18.6%) were the most frequently identified pathogens [39]. In this study, PRRSV (68.9%), *Mycoplasma hyorhinis* (48.7%), *Streptococcus suis* (31.1%) and *Glaesserella parasuis* (26.8%) were the most frequently isolated pathogens co-infected with *Pasteurella multocida* (Table 5). *Pasteurella multocida* is generally considered as a secondary bacterial pathogen, and PRRSV-induced impaired phagocytosis of porcine alveolar macrophages (PAMs) may lead to lung damage favouring the development of secondary bacterial infections [40]. A study showed that positive rates of PRRSV were significantly correlated with those of *Mycoplasma hyorhinis* in the field swine [41]. In the study, 64.6% (73/113) of PRRSV-positive samples were co-infected with *Mycoplasma hyorhinis*, and 44.5% (73/164) of the samples were combined infection of PRRSV, *Mycoplasma hyorhinis* and *Pasteurella multocida*. *Mycoplasma hyorhinis* may affect the immune response by inducing the cytotoxicity of immune cells and/or the secretion of cytokines [42].

Conclusions

Our findings have demonstrated that the serovar prevalence of *Pasteurella multocida* strains isolated in Taiwan is consistent with other findings in the world. The MLST analysis has shown that ST10 is the major genotype of the *Pasteurella multocida* strains carrying *toxA* gene. Further *Pasteurella multocida* challenge studies are warranted to verify the relationship between virulence genes and the pathogenicity. Florfenicol is often used for the prevention or treatment of respiratory diseases in pigs. However, the *Pasteurella multocida* isolates in this study showed lower sensitivity to florfenicol. Also, this study found a significant association between *floR* gene and florfenicol resistance, aligning with the findings of most previous studies. The antimicrobial susceptibility tests have demonstrated that cephalosporins (cefazolin and ceftiofur) and quinolones (flumequine and enrofloxacin) exhibit good sensitivity against *Pasteurella multocida* isolates. In clinical practice, it is advisable to prioritize the use of these two classes of drugs for treatment of *Pasteurella multocida* infection. Taken together, this study updates the information on the molecular epidemiology of *Pasteurella multocida* in Taiwan and the antimicrobial susceptibility profiles of *Pasteurella multocida* isolates collected from diseased pigs, providing guidance for on-site veterinarians on management and control of pig diseases.

Methods

Bacterial isolation and identification

From June 2017 to May 2020, 161 diseased pigs in 155 herds were submitted to the Animal Disease Diagnostic Centre (ADDC), Department of Veterinary Medicine of National Chiayi University (Taiwan) for necropsy. A total of 164 isolates were collected from lungs and nasal cavities of these diseased pigs. Each sample was cultured on 5% sheep blood agar (BBLTM Blood agar base infusion; BD, USA) and MacConkey agar (DifcoTM MacConkey agar; BD, USA), and then incubated under aerobic conditions at 37 °C for 18–24 h. Suspected *Pasteurella multocida* samples, which could not grow on MacConkey agar, were identified as ash grey and viscous colonies without hemolysis on blood agar.

Genomic DNA of each suspected isolate was extracted using Biokit® Genomic DNA Purification Kit (BioKit®, Taiwan) and used for PCR amplification of *kmt1* gene

[43]. The PCR product was purified and sequenced to identify *Pasteurella multocida* using BLAST (http://blast.ncbi.nlm.nih.gov/). The *Pasteurella multocida* capsule serovars (serovar A, B, D, E, F), LPS serovars (L1-L8) and the presence of virulence gene *toxA* and antibiotic resistance gene *floR* were determined using the DNA sample of each *Pasteurella multocida* isolate. Primer sequences were designed according to previous literatures [12, 13, 44, 45].

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested quantitatively by broth microdilution method on Mueller–Hinton II broth (Difco Laboratories, Detroit, Michigan, USA) supplemented with 5% fetal bovine serum according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018) [46]. The *Pasteurella multocida* strains grew overnight in Mueller Hinton II broth (BBL™, USA) at 37 °C. A bacterial suspension equal to a 0.5 McFarland standard was prepared in 0.85% saline by using a turbidity meter (Dade International Inc., West Sacramento, CA). A total of 14 antimicrobial agents frequently administered to treat swine respiratory disease, including ampicillin, cefazolin, ceftiofur, doxycycline, enrofloxacin, erythromycin, florfenicol, flumequine, kanamycin, lincomycin, lincospectin, tylosin, tiamulin and trimethoprim/sulfamethoxazole, were used for the test. Test concentrations were set between 0.125 ~ 1024 μg/mL. The minimum inhibitory concentrations (MICs) were determined after 18–24 h of incubation at 37 °C. The quality control strains used in the study were *Haemophilus influenzae* (ATCC 49247), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Pasteurella multocida* (ATCC 12947) and *Staphylococcus aureus* (ATCC 29213) (CLSI, 2018) [46].

Multilocus sequence typing analysis

Seven housekeeping genes including *adk*, *aroA*, *deoD*, *gdhA*, *g6pd*, *mdh*, and *pgi* were selected for the MLST assay via literature survey [47]. The confirmed PCR products were then sent to Tri-I Biotech, Inc. (Taiwan) for 5' to 3' and 3' to 5' sequencing by dye terminator sequencing method on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, USA). Subsequently, the sequencing results were uploaded to BioNumerics® version7.6.3 (Applied Maths, USA), and then the alleles and genotypes were compared with the PubMLST database (https://pubmlst.org/organisms/pasteurella-multocida/) to obtain the allelic profile of each isolate and to assign the respective ST. The obtained STs were compared with the ones in the multi-host MLST database on PubMLST by using the based upon related sequence type (BURST) algorithm, which partitioned the *Pasteurella multocida* strains. When six of the seven loci share the same allelic number, the STs were clustered. Minimum spanning tree (MST) was generated using BioNumerics® version 7.6 software (Applied Maths, USA), with a distance set to ≤ 1 for partitioning. This approach grouped nodes and their associated subtrees into clonal complexes [48].

Statistical analysis

Student's t-test analysis was conducted to compare the MIC results between strains with and without *floR* gene, and the MIC results among strains of different serovars. The *p*-values were calculated, where $p < 0.05$ indicates a significant difference between the two groups. Association was determined by Chi-square test. $P < 0.05$ indicates a significant association.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04595-1.

Supplementary Material 1

Supplementary Material 2

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Not applicable.

Authors' contributions

Hong-Chi Kuo designed research; Che-Cheng Liao collected samples; Ching-Fen Wu, Che-Cheng Liao and Hong-Chi Kuo performed research, analyzed data and wrote the draft manuscript. Ching-Fen Wu, Chi-Chung Chou, Chao-Min Wang, Szu-Wei Huang and Hong-Chi Kuo edited and reviewed the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated during the current study are available in the PubMLST repository [https://pubmlst.org/bigsdb?db=pubmlst_pmultocida_isolates&page=query&prov_field1=f_country&prov_value1=Taiwan&submit=1], and the NCBI repository (BioProject number: PRJNA989137) [https://www.ncbi.nlm.nih.gov/bioproject/].

Declarations

Ethics approval and consent to participate

No ethics commission approval for this study was required as all the diseased pigs were sent to Animal Disease Diagnostic Center in National Chiayi University by the farm owners for necropsy, and process including tissue and fluids collection and further bacterial isolation and identification were routine exams for disease diagnosis and treatment.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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