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SHORT COMMUNICATION

Molecular characterisation and antibiotic sensitivity profile of *Pasteurella multocida* isolated from poultry farms in Malaysia

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ABSTRACT. Fowl cholera has caused significant economic losses in many poultry producing countries worldwide. In Malaysia, outbreaks of fowl cholera are frequently reported and encountered in different types of poultry productions. The objective of this study was to characterise 13 avian *Pasteurella multocida*, isolated from fowl cholera outbreaks in Central Peninsular Malaysia in the period between 2000 and 2018. The isolates were subjected to multiplex polymerase chain reaction (PCR) for capsular serotyping, disc diffusion method for antimicrobial susceptibility profiles, and molecular genotyping using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The capsular serotyping showed all 13 *Pasteurella multocida* isolates belonging to capsular serotype A. The antimicrobial susceptibility showed several multidrug resistance strains among the *P. multocida* isolates. All the isolates were resistant to erythromycin (100%), streptomycin (68%), tetracycline (37%), enrofloxacin (37%), florfenicol (23%), penicillin G (14%), gentamicin (14%), and amoxicillin (14%). The PFGE analysis clustered the isolates into three clones. Group A included isolates with a similarity of 87% from the year 2000, 2013, and 2018. Three sequence types were identified using MLST typing namely, ST129, ST231, and ST355. The ST355 was assigned for the first time in the Rural Industries Research and Development Corporation (RIRDC) database. Besides, ST129 has been reported in India, China, and Sri Lanka, which highlights the possibility of transmission between Asian countries. This study provides an insight into epidemiological information of *Pasteurella multocida* that causes fowl cholera outbreaks in the central region of Peninsular Malaysia.

Key words: antimicrobial susceptibility, MLST, *Pasteurella multocida*, PFGE.

INTRODUCTION

Pasteurella multocida (*P. multocida*) is a Gram-negative bacterium that can cause a wide range of diseases in animals, such as fowl cholera in poultry, haemorrhagic septicemia in bovine, and atrophic rhinitis in swine (Wilkie *et al* 2012, Wilson and Ho 2013). Fowl cholera is an epizootic, highly contagious avian disease that could affect several avian species including commercial chickens (Botzler 1991). Since it was discovered, it has caused significant economic losses in the poultry industry worldwide (Harper *et al* 2006). The disease may occur as an acute septicaemia form with high morbidity and mortality (up to 100%), or as a localised chronic infection (Heddleston *et al* 1964, Harper *et al* 2006). Sudden death for a large number of birds is usually the first clinical sign in acute fowl cholera (Glisson *et al* 2013). *Pasteurella multocida* can currently be subdivided into four subspecies: subsp *multocida*, subsp *gallicida*, subsp *septica*, and subsp *tigris*. All subspecies, excluding tigris, have been isolated from fowl cholera outbreaks (Harper *et al* 2006). Serotype A is the dominant serotype of *P. multocida* causing fowl cholera while serotypes B, D, and F have been less reported to cause disease in poultry (Wilkie *et al* 2012). Outbreaks were reported in Asia and all over the world (Wang *et al* 2013, Jones *et al* 2013, Singh *et al* 2013). Antibiotics are widely used in the treatment of *P. multocida* infections in poultry, which have increased antibiotic resistance (Murray 1992). A study conducted in Brazil showed 19.64 % of *P. multocida* strains isolated from chicken and turkey farms were multidrug-resistant to three or more drugs in different categories using the disc diffusion method (Furian *et al* 2016). A number of epidemiological studies were conducted to investigate the distribution of *P. multocida* strains in several countries (Sarangi *et al* 2016, Li *et al* 2018, Peng *et al* 2018). Pulsed-field gel electrophoresis (PFGE) is a genotyping technique that analyses bacterial chromosomes using restriction enzyme into DNA fragments. The PFGE fragments pattern can be used to study the strain variation and evolution (Gunawardana *et al* 2000). PFGE also has been used to study outbreaks of fowl cholera in poultry (Kardos and Kiss 2005, Sellyei *et al* 2017). However, multilocus sequence typing (MLST) is the current gold standard typing method for *P. multocida*, which uses seven housekeeping genes to characterise and study the global distribution of *P. multocida* sequence types (STs) (Kardos and Kiss 2005, Subaaharan *et al* 2010, Singh *et al* 2013).

In Malaysia, avian *P. multocida* outbreaks were frequently reported in commercial and backyard farms (Arumugam *et al* 2011, Nafizah *et al* 2014, Khoo *et al* 2017). However, information on *P. multocida* serogroups, antibiotic resistance profile, and molecular genotyping are poorly investigated. Therefore, the aim of this study is to molecular characterise *P. multocida* isolates from fowl cholera outbreaks submitted to the Laboratory of Bacteriology at Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia. The isolates undergo multiplex

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PCR serotyping to determine the serogroup as well as the disc diffusion method to determine the resistance profile of the isolates. Additionally, genotyping was performed using PFGE and MLST to study the variation and evolution of the *P. multocida* isolates.

MATERIAL AND METHODS

BACTERIAL ISOLATES

Thirteen *P. multocida* subspecies *multocida* isolates from the Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia were analysed in this study (table 1). The samples were submitted for diagnostic purposes, between the years 2000 to 2018 from fowl cholera outbreaks in backyard chicken farms located in Selangor, Malaysia. The bacteria isolates were obtained from chicken internal organs including liver, spleen, and lungs, and were subject to biochemical identification. The samples were cultured onto 5% blood agar (OXOID, UK). Suspected colonies showing *P. multocida* colony morphology, were subjected to biochemical tests, namely, oxidase, indole, Triple Sugar Iron (TSI), Sulfide Indole Motility (SIM), citrate, urease reactions and Ornithine Decarboxylase Test (ODC), trehalose, mannitol, D-sorbitol, and dulcitol.

MOLECULAR IDENTIFICATION

Genomic DNA was extracted using the boiling method. The bacteria were boiled at 97 °C for 10 min, then placed in an ice container for 5 min, then centrifugated for 10 min at room temperature. The isolates were confirmed as *P. multocida* using PCR targeting the KMT1 gene as described by Townsend *et al* (1998). *P. multocida* ATCC 12945 was used as a positive control.

MULTIPLEX PCR CAPSULAR TYPING

The isolates were subjected to capsular serotyping using the primers designed by Townsend et al (2001). Genomic DNA was extracted using the boiling method. The multiplex PCR was carried out with a final reaction volume of 50µl, 30 PCR cycles; 95°C for 45 sec, 56.2°C for 45 sec, 72°C for 45 sec. The PCR product was visualised using a UV transilluminator.

ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility test was carried out using the disc diffusion method following the Clinical and Laboratory Standards Institute standards (CLSI) VET01- A4 (4th ed.) and the M45 (3rd ed.). Two replicates were performed for each isolate against eight antibiotics, namely, streptomycin 10µg, amoxicillin 10µg, tetracycline 30µg, gentamicin 10µg, erythromycin 15µg, penicillin G 10u, enrofloxacin 5µg, and florfenicol 30µg. The bacteria suspensions were cultured on Mueller-Hinton agar then the antibiotic where placed on the plates. After 24 hours of incubation, the average zone of inhibition was measured and interpreted. The *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control.

Table 1. Antibiotic resistance profile for each *P. multocida* isolated from fowl cholera outbreaks in poultry farms in Malaysia in the period of 2000 to 2018. *Pasteurella multocida* isolated from fowl cholera outbreaks in poultry farms in Malaysia in the period of 2000 to 2018.

Number	Year	Sample	PFGE	ST	Antibiotics resistance profile ‡)
1	2000	PM201	A3	129	E
2	2000	PM202	A3	-	E
3	2000	PM203	A3	-	E
4	2000	PM204	A3	-	E
5	2013	PM205	A1	129	ST, ENR, TE, E
6	2013	PM206	A2	-	ST, ENR, TE, E. AMX, CN, P
7	2014	PM207	B	129	ST, ENR, TE, E, AMX, CN, P, FFC
8	2016	PM208	C1	355	ST, E
9	2016	PM209	C2	-	ST, E
10	2016	PM210	C3	-	ST, E
11	2016	PM211	C3	-	E
12	2018	PM212	A1	-	ST, ENR, TE, E, FFC
13	2018	PM213	A1	231	ST, ENR, TE, E, FFC

‡) List of antibiotics the isolates were resistant against, according to M45 (3rd ed.) and VET01- A4 (4th ed.) of the CLSI standard. ST= Streptomycin, AMX= Amoxicillin, TE= Tetracycline, CN= Gentamicin, E= Erythromycin, P= Penicillin G, ENR= Enrofloxacin, FFC= Florfenicol.

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

A single colony from each isolate was cultured in brain heart infusion broth (BHI) (OXOID, UK) and incubated at 37°C for 24 hr. The culture was then mixed to cell suspension buffer and adjusted to 0.6-0.7 of McFarland standard. The cell mixtures were pipetted into CHEF disposable plug moulds (Bio-Rad Laboratories, USA) and allowed to solidify for 10 min at 4 °C. The plugs were transported into 2 ml cell lysis buffer and incubated in a water bath at 56 °C for 17 hr with one hour shaking at 100 rpm. The plugs were washed two times with deionised water for 10 min each at 50 °C, followed by 5 times washing with the TE buffer. A small slice of each plug was placed in a 2 ml tube containing 200 µl per-restriction mixture for 15 min at 37 °C. Then, a restriction mixture containing the ApaI enzyme was added and incubated at 37 °C for 2 hr. Finally, the restriction mixture was removed, and 0.5 TBE buffer was added for 5-10 min. The electrophoresis was performed using the following conditions; initial switch time 1 sec, final switching time 40 sec, a constant voltage of 6 V, and an angle of 120. With a total running time of 23 hr and 14°C running temperature. BioNumerics 6.6 software was used to analyse the *P. multocida* PFGE profiles. The dendrogram was created with 1% optimization and 1% tolerance using the Dice similarity coefficient, by unweighted paired group method of arithmetic averages (UPGMA). Strains with more than three differences in DNA fragments and a similarity of <85% were classified into different PFGE types (Van Belkum *et al* 2007).

MULTILOCUS SEQUENCE TYPING

One isolate representing each year was characterised via the MLST scheme. Briefly, PCR amplification was carried out for seven housekeeping genes, using the primers designed by Subaaharan *et al* (2010), then the sequences were analysed using the *P. multocida* MLST RIRDC database[1] to get the *P. multocida* sequence type (ST).

RESULTS AND DISCUSSION

Pasteurella multocida is a significant pathogen that causes epizootic diseases in animals including fowl cholera in poultry. Outbreaks of fowl cholera have been reported all over the world in both wild and domestic birds (Botzler 1991, Kardos and Kiss 2005). To enhance our understanding of the variation and transmission of the bacterial strains, many techniques were used to study the population structure of *P. multocida* worldwide (Gunawardana *et al* 2000, Subaaharan *et al* 2010). In this study, thirteen isolates confirmed to be *P. multocida* were found to belong to capsular serotype A.

The isolates showed an overall high level of antibiotic resistance, five isolates were multidrug resistance (MDR) (PM205, PM206, PM207, PM212, and PM213), as shown in table 1. Also, the PM207 isolate was found to be resistant to all antibiotics tested, namely erythromycin, streptomycin, tetracycline, enrofloxacin, florfenicol, penicillin G, gentamicin, and amoxicillin, which belonged to six classes of antibiotics. MDR strains of *P. multocida* have been reported in the USA, Germany, Spain, and recently reported in China MDR strains of *P. multocida* against florfenicol, chloramphenicol, ofloxacin tetracycline streptomycin, kanamycin, and sulfamethoxazole (Li et al 2018; Zhu et al 2020). Overall, the isolates were resistances to erythromycin (100%), streptomycin (68%), tetracycline (37%), enrofloxacin (37%), florfenicol (23%), penicillin G (14%), gentamicin (14%), and amoxicillin (14%). Similar findings were reported in a study of avian *P. multocida* in Mississippi, whereby the isolates were resistant 78% against erythromycin, 78% streptomycin, and 46% penicillin (Jones et al 2013). In this study, the high resistance against erythromycin and streptomycin was reported. It is high likely that these occur due to excessive usage of antibiotics in poultry farms in Malaysia. In addition, these antibiotics are also used as growth promoter in poultry feeds in Malaysia (Hassali et al 2018). Besides, *P. multocida* isolates may also have acquired resistance genes from other Gram-negative bacteria (Wilson and Ho 2013).

The PFGE phylogenetic tree clustered the isolates into three groups shown in table 1. Group A has an 87.1% similarity among isolates from different years (2000, 2013, and 2018). Group B showed 85% similarity, including isolates from the year 2016. PFGE type C has one isolate that belongs to the year 2014, as shown in figure 1. The high similarity of 87.1% in group A suggests that these isolates were isolated from related outbreaks of the same strains of *P. multocida* and had developed or acquired resistant genes from other Gram-negative bacteria over the years. For instance, PFGE was used to investigate the epidemiology of strains *P. multocida* isolated from two cases of fowl cholera outbreaks in the region of eastern Hungary. The results showed high genetic relatedness among the isolates and suggested that the second outbreak was recurrent of the same strain of *P. multocida* (Kardos and Kiss 2005).

MLST typing is the gold standard typing method for *P. multocida* (Subaaharan *et al* 2010). Five isolates were typed using MLST, and three STs were detected, namely, ST129 from three isolates, ST231, and ST355, as shown in table 1. Besides, ST355 was identified for the first time in the MLST database in this study. Interestingly, ST129 was recently reported in several epidemiological studies in Asia. For instance, a study was published in 2013 in China reported many outbreaks of fowl cholera involving *P. multocida* ST129 (n=40) (Wang *et al* 2013). Moreover, another study in 2016 in China reported a high occurrence (91%) of ST129 as well (Wang *et al*

[1] http://pubmlst.org/pmultocida_rirdc/

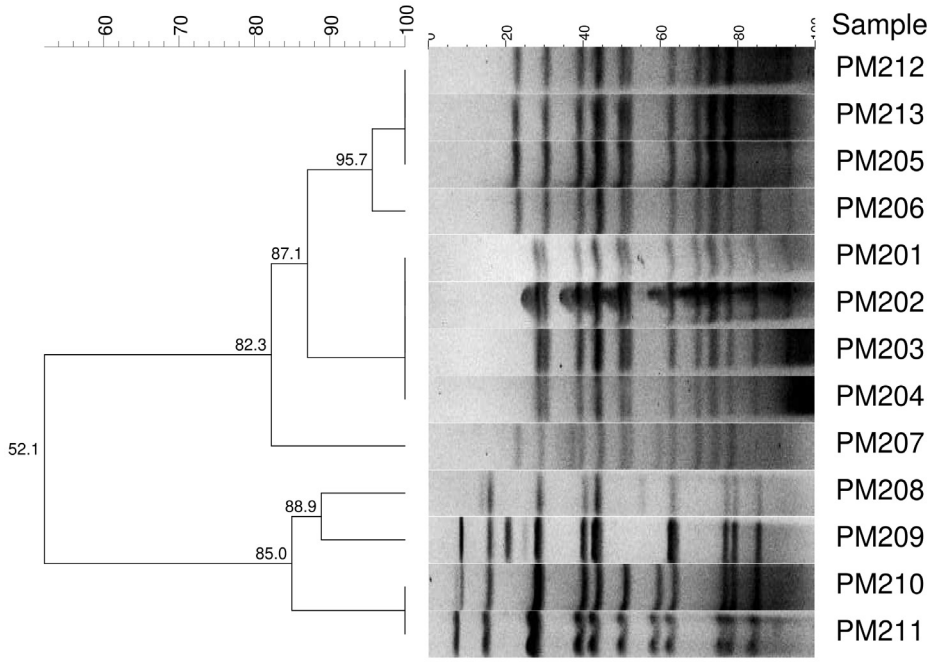

Figure 1. PFGE phylogenetic tree of *P. multocida* isolates using BioNumerics 6.6. The dendrogram shows high similarity (82.3%) between the years (2000, 2013, 2014, and 2018) and high similarly among the isolates from the year 2016.

2016). A recently published study in China reported that 84% of avian *P. multocida* isolates were ST129, and they suggested that ST129 is a significant and high virulence ST of *P. multocida* in southwestern China, causing fowl cholera infections in many types of poultry species (Li *et al* 2018). The study also found that most of the ST129 isolates exhibited multidrug resistance for antibiotics, including amoxicillin, tetracycline, florfenicol, and streptomycin, which is very similar to the resistance pattern findings for the ST129 isolates in this study (table 1). Furthermore, *P. multocida* ST129 was also reported in India in fowl cholera outbreaks (Sarangi *et al* 2016). And in Iran from chicken fowl cholera outbreak according to the RIRDC MLST database[2]. On the other hand, the ST129 was also reported to cause hemorrhagic septicemia in bovine in Sri Lanka (Hotchkiss *et al* 2011), as well as infection in pigs according to the RIRDC MLST database[3]. As a result, ST129 was suggested as adaptable to many types of hosts (Hotchkiss *et al* 2011, Peng *et al* 2018). Although the distribution of the ST129 was discovered in India, it was suggested as a result of possible transmission from China and Sri Lanka (Sarangi *et al* 2016). In the current study, ST129 was identified in several fowl cholera cases among poultry in Malaysia and had a relatively similar antibiotic profile to the ST129 isolates reported in southwestern China (Li *et al* 2018). This finding shows the high possibility of transmission of ST129 from or to China into Malaysia. Besides, *P. multocida* was proved to spread between different countries. In a study of the fowl cholera outbreaks in Denmark and Sweden, it shows that migrating birds had spread a strain of *P. multocida*, which caused several fowl cholera outbreaks among these countries (Christensen and Bisgaard 2000, Petersen *et al* 2001). Regarding our study, Malaysia is a significant winter home for many species of migratory birds coming from the north due to its stable weather (DeCandido *et al* 2004). Thus, increasing the possibility of transmission of new strains into the country.

MLST is an effective tool to study *P. multocida* genotype variation and evolution (Subaaharan *et al* 2010). *Pasteurella multocida* ST231 belongs to the CC129 and shared six alleles (*est, pmi, Zwf, mdh, gdh, pgi*) with ST129 single locus variant (SLV) besides sharing a relatively similar antibiogram (table 1), which suggested that it evolved from ST129. Another study had published a complete genome sequence of *P. multocida* serotype A, isolated from haemorrhagic septicemia infection in buffaloes in Malaysia under the accession No. CP007205 in NCBI GenBank (Jabeen *et al* 2017). Based on the analysis of the RIRDC MLST database, this strain belongs to ST201, which also belongs to CC129. This isolate is SLV with ST129, this finding suggested that it evolved from ST129 and caused haemorrhagic septicemia infection in buffaloes

[2] https://pubmlst.org/bigsdb?page=info&db=pubmlst_pmultocida_isolates&id=984

[3] https://pubmlst.org/bigsdb?db=pubmlst_pmultocida_isolates&l=1&page=profiles

in Malaysia. Haemorrhagic septicemia was reported to be endemic and of significant economic importance in many countries, including Malaysia (Benkirane and De Alwis 2002). The investigation provides significant insights into the epidemiological importance of CC129 in Malaysia. In contrast, recently published in China, there is a report on a double-locus variant (DLV) genotype from the CC129 identified as the first hypervirulent and multi-antimicrobial resistant avian *P. multocida* (ST342) (Zhu *et al* 2020). In India, SLV from CC129 (ST280) was also reported causing fowl cholera outbreaks (Sarangi *et al* 2016). These findings highlight the threat of *P. multocida* CC129 as ubiquitous and causing infections in many animal host species in Asian countries.

Overall, this study provides important epidemiological data on the diversity of *P. multocida* causing fowl cholera infection in Malaysia and highlighted the high potential of transmission of the same sequence type among the nearby countries. This study also reported the CC129 is a big threat to the poultry industry in Malaysia, and as a wildly reported CC causing fowl cholera in south Asian countries. The high antibiotic resistance shown among isolates in this study warrants the prudent use of antimicrobial agents in the poultry production in Malaysia.

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