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Multilocus Sequence Typing of *Pasteurella multocida* Isolates from Acute Fowl Cholera Outbreak in Layer

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ABSTRACT Fowl cholera is an infectious disease caused by *Pasteurella multocida* that contributes to high economic loss in the commercial chicken industry. Three *Pasteurella multocida* strains were isolated from outbreaks of acute fowl cholera in the Korean layer farms from 2018 to 2019. One strain was identified and serotyped using capsular PCR typing. This strain was also genotyped by lipopolysaccharide (LPS) PCR typing as A: L3, whereas other strains were non-typable. The multilocus sequence typing (MLST) result showed that the A: L3 strain is sequence type (ST) 134; the non-typable strains were recorded as the following new STs: ST 366 and ST 374. Using phylogenetic tree analysis based on MLST sequences, we determined that ST 366 and ST 374 are closely related to the reference strains that were previously isolated from duck and chicken in Korea, and they were highly prevalent within the Korean cluster. In conclusion, *Pasteurella multocida* strains were identified and isolated in this study. Furthermore, this is the first report of using MLST to determine the prevalence of fowl cholera in Korea.

(Key words: *Pasteurella multocida*, acute fowl cholera, MLST, prevalence)

INTRODUCTION

Pasteurella multocida (PM) is a common bacterial pathogen which infected on a wide range of animals like cattle, swine, chicken, duck, rabbit, and human (Harper et al., 2006). In poultry, PM causes fowl cholera disease with high morbidity and mortality on domestic and wild birds (Subaaharan et al., 2010). The conventional subgroup typing based on the capsular and somatic antigen classified this bacteria into 5 capsular types (A, B, D, E, and F) and 16 somatic serotypes (Carter, 1955; Heddleston et al., 1972). Recently, the multiplex capsular and lipopolysaccharide polymerase chain reaction (PCR) assay were developed as an alternative conventional serotyping test (Townsend et al., 2001; Harper et al., 2015). However, the correlation between the serotyped strains has not been determined. Moreover, some isolated strains were not identified by the traditional serotyping method (Wilson et al., 1993; Singh et al. 2013). So, the multi-locus sequence typing (MLST) for PM was developed and used as the standard genotyping method for epidemiological study (Subaaharan et al., 2010).

In Korea, fowl cholera is a rare disease with both acute and chronic clinical types. PM isolates were identifed from outbreaks in the poultry farm and water-fowl (Kwon et al., 2003; Woo et al., 2006). In the previous study, the PM strain isolated from wild bird was different from the PM strains isolated from broiler breeder (Woo et al., 2006). As the result, the epidemiological information of PM strains affecting the domestic bird is still limited. Recently, phenotypic characterization based on MLST of PM isolated from pigs and rabbit in Korea have been reported (Jeong et al., 2018; Oh et al., 2019). In this study, we confirmed and characterized PM isolates from acute fowl cholera outbreaks in layer chicken using serotyping and MLST.

MATERIAL AND METHOD

1. Bacterial Isolation

Three acute fowl cholera outbreaks from different layer farms were identified at Avian Disease Laboratory, Chungbuk National University during 2018~2019. The first outbreak occurred in Daegu area in June 2018 with constant mortality

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of 20 birds per day for 2 weeks. In the second and the third outbreak, farms located in proximity in Chungbuk province (Jecheon-si and Danyang-kun) were infected in November 2018 and September 2019. Chickens were found dead without any clinical sign in all cases. The PM strains were isolated from tissue samples including trachea, liver, spleen, and ovary follicle. The bacterial isolates were grown on blood agar (Synergy Innovation co., Korea) at 37℃ for 24 h. The single colony from liver was cultured in the Tryptic soy broth and incubated overnight at 37℃ for further process.

2. Molecular Identification

The DNA of bacteria was extracted using the Patho Gene-spin DNA/RNA extraction kit (iNtRON bio., Korea). A pair of primers, KMT1T7 and KMT1SP6, amplifying 460bp gene fragment was used to confirm all PM isolates and followed PCR temperature condition described by Townsend et al. (1998).

3. Multiplex PCR Typing

The isolated PM strains were typed using the multiplex capsular and lipopolysaccharide PCR with primers described in the previous study (Townsend et al., 2001; Harper et al., 2015).

4. Multi-locus Sequence Typing

The MLST scheme developed by Subaaharan et al. (2010) based on the seven housekeeping genes was performed on the isolates following the protocol at RIRDC MLST database (http://pubmlst.org/pmultocida_rirdc/). The sequences were submitted to MLST database to identify STs. To analyze the phylogenetic data of these PM isolates, previously identified PM isolates were included such as ST13, ST44, ST50, ST74, ST122, ST286, ST347 (swine-Korea) ST8, ST351, ST352, ST353, ST368 (avian-Korea), ST359, ST360, ST361, ST362 ST363, ST364 (feline-Korea) ST365 (rabbit-Korea), ST129, ST20, ST1, ST2, ST35, ST37 (global avian) and ST123 (bovine). Phylogenetic tree of concatenated DNA sequences was analyzed using the neighbor-joining method with 1,000 bootstrap replicates by the software MEGA version 7.0 (http://www.megasoftware.net).

RESULTS

1. PM Isolates

The submitted cases were described with sudden death with over 20 layer chickens per day. In the necropsy, the main gross lesions were multifocal necrosis in the liver and ruptured follicles. The bacterial colonies were grown on the blood agar but not on the Macconkey agar from the liver, trachea, and ovarian follicle samples. The pure colony was further analyzed and confirmed as *P. multocida* based on 460bp PCR amplicon.

2. PM Serotyping

From the results of the multiplex capsular PCR typing, only ADL18 1033 isolate was classified as serogroup A (Table 1). The multiplex lipopolysaccharide PCR identified this isolate belonged to the L3 genotype. In contrast, the serotype of ADL18 2436 and ADL19 1915 were not determined with no amplification PCR observed.

3. MLST Genotyping

The serogroup A isolate was characterized as ST134 which

Table 1. Characteristics of the *Pasteurella multocida* isolates used in this study

Strain	Age (w.o)*	Isolation year	Province	Source	Capsular serogroup	Serotype	MLST
ADL18 1033	44	2018	Daegu	Liver	A	L3	ST134
ADL18 2436	25	2018	Chungbuk	Liver	-**	-	ST366
ADL19 1915	39	2019	Chungbuk	Liver	-	-	ST374

* weeks old.
** undetermined.

previously isolated from the respiratory tract of bovine in France (Hotchkiss et al., 2011). Two unidentifed capsular serotype isolates (ADL18 2436 and ADL19 1915) were listed as new sequence typing ST366 and ST374, respectively. Phylogenetic tree analysis showed the close relationship between ST366 and ST374 with ST353 and ST352, mainly isolated from duck in Korea (Fig. 1). The avian isolates did not share any STs with swine, rabbit, or feline isolates in Korea.

DISSCUSION

Pasteurella multocida is a Gram-negative coccobacillus bacteria causing several animal diseases of significant economic impact to domestic industries over the world including fowl cholera in poultry, atrophic rhinitis in swine and hemorrhagic septicemia in cattle (Harper et al., 2006). In Korea, the acute fowl cholera was mostly reported from wild birds, while chronic fowl cholera is common on domestic chicken

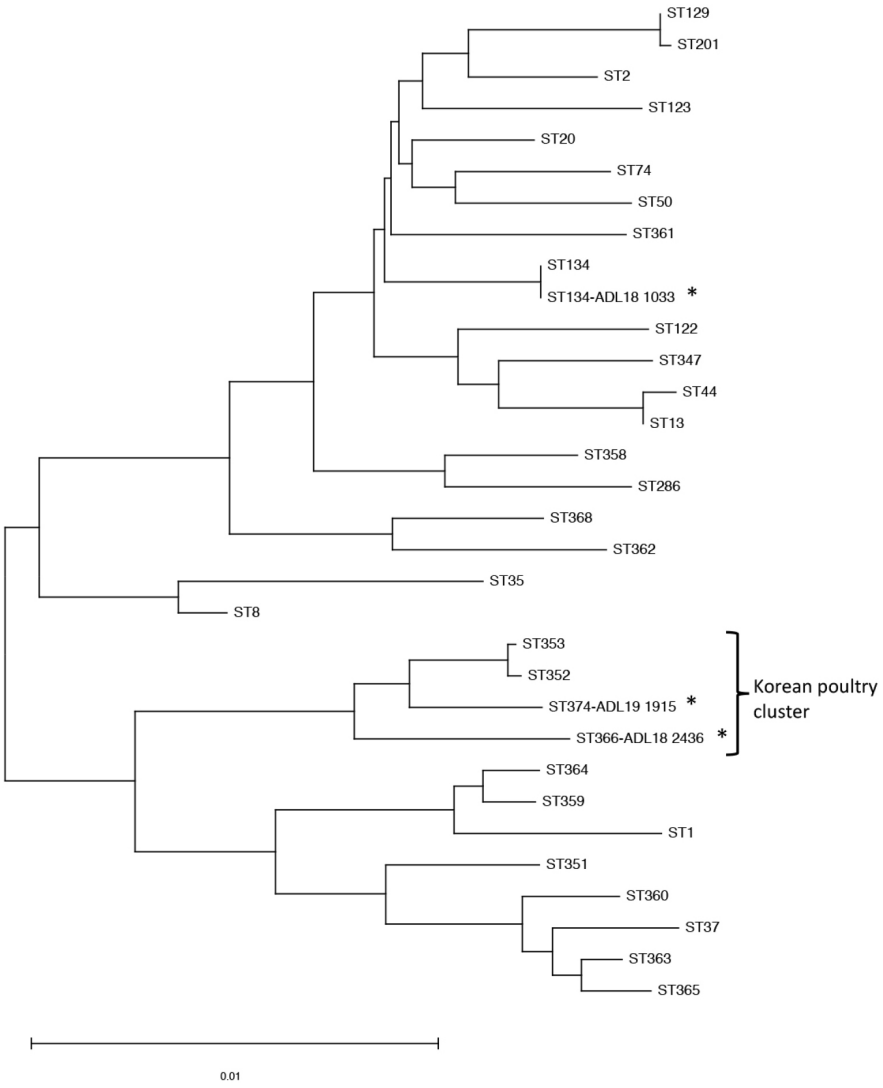

Fig. 1. Phylogenetic tree of different *Pasteurella multocida* sequence type. The concatenated DNA sequences of 7 housekeeping genes were analyzed based on Neighbor-joining method with 1,000 bootstrap replicas. Gene sequences were submitted to RIRDC MLST database (http://pubmlst.org/pmultocida_rirdc/).

* The identified isolates from layer in this study.

(Kwon et al., 2003; Woo et al., 2006). Additionally, the acute fowl cholera is commonly reported in the low biosecurity duck meat farm, but it is rarely reported from the layer with limited bacterial source information. In this study, three recent cases from layer farms were determined with the typical clinical sign and gross lesion of acute fowl cholera consisting of high mortality of sudden death and multifocal necrosis in the liver. Although there is no demonstration, the wild bird and rat may act as carriers to introduce PM into the farms. The case of layer farm in Deagu reported with only 1 of 4 farmhouses affected with the bacteria. This farmhouse maintained the old system and located next to a temporary waste disposal site where has the appearance of sparrows and rats.

Among five capsular serogroups A, B, D, E, and F, fowl cholera is known to be associated with the most prevalent of the capsular serogroup A and somatic serotypes 1, 3, and 4 in birds. In this study, only one PM isolate was determined to be a member of serogroup A:L3. The serotypes of two isolates from Chungbuk were not available to determine based on the capsular and LPS serotyping PCR. The result indicated that the isolate from the farm in Daegu was not related to the isolates from farms in Chungbuk.

For further characterization of these isolates, MLST based on seven loci was used to investigate molecular epidemiology. Since the development of RIRDC MLST for PM isolates in poultry by Subaaharan et al. (2010), it has been widely applied to study the genetic diversity of PM strains isolated from variant animals. The *Pasteurella multocida* MLST database is a useful tool that enables to share isolates details and provides scope to study genetic and epidemiology of fowl cholera over the world. Phylogenetic tree analysis of concatenated sequences showed the close relation of the new STs to the STs isolated from the duck in Korea, suggesting the high possibility of the same source of these isolates. The ST of duck isolates were uploaded to the MLST database by Animak and Plant Quarantine Agency, South Korea. The four STs, including ST352, ST353, ST366, and ST374, were only reported from Korean poultry and had a highly different sequence from the other STs. They arranged into a cluster of dominant isolate, causing the fowl cholera in the poultry in Korea.

MLST genotyping could show the same *Pasteurella multocida* STs between the different animal host such as poultry, pig and cattle; cat and chicken (Wang et al., 2013; Singh et al., 2014). Recently, there were several studies to determine PM associated with swine, rabbit, and feline in Korea (Jeong etal., 2018; Oh et al., 2019). Nevertheless, there was no evidence of genotype relevance between the isolates in this study or the poultry isolates from Korea with the pig, feline, and rabbit. The cattle could be the source of acute fowl cholera when one isolate have the same ST134 with an isolate previously identified in bovine (Hotchkiss et al., 2011). The result suggested ST134 is a globally distributed strain and can be associated with different host. The population structure of *Pasteurella multocida* is improved by MLST as more isolates are added to the database such as ST129, ST8 and ST9 (Singh et al., 2013; Wang et al., 2013). However, the information of sharing the PM is limited due to the few number of isolates included in this study and the lack of study on the PM in the cattle in Korea.

SUMMARY

In this study, we have characterized and typed the unfrequent isolates of *Pasteurella multocida* in layer chicken in Korea using molecular methods. The phenotyping and genotyping methods based on the capsular and lipopolysaccharide were unable to classify the isolates with only one of three strain was determined as A: L3. The MLST genotyping result showed the sharing ST of strain A: L3 with bovine strain in France and the correlation between two new STs with the ST from duck in Korea. These strains were classified into the Korean poultry dominant cluster, which is different from swine, cat, and global strains. MLST method and shareable database provided valuable information to understand the epidemiological property of PM in poultry in Korea.

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REFERENCES

Carter GR 1955 Studies on *Pasteurella multocida*. I. A hemagglutination test for the identification of serological types. Am J Vet Res 16(60):481-484.

Harper M, Boyce JD, Adler B 2006 *Pasteurella multocida* pathogenesis: 125 years after Pasteur. FEMS Microbiol Lett 265(1):1-10.

Harper M, John M, Turni C, Edmunds M, Michael FS, Adler B, Blackall PJ, Cox AD, Boyce JD 2015 Development of a rapid multiplex PCR assay to genotype *Pasteurella multocida* strains by use of the lipopolysaccharide outer core biosynthesis locus. J Clin Microbiol 53(2):477-485.

Heddleston KL, Gallagher JE, Rebers PA 1972 Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. Avian Dis 16(4):925-936.

Hotchkiss EJ, Hodgson JC, Lainson FA, Zadoks RN 2011 Multilocus sequence typing of a global collection of *Pasteurella multocida* isolates from cattle and other host species demonstrates niche association. BMC Microbiol 11(1):115.

Jeong J, Lee K, Choi EJ, Kim HY, Sohn JH, So B, Jung JY 2018 Meningoencephalitis and pneumonia caused by *Pasteurella multocida* in rabbits. Korean J Vet Res 58(1): 61.

Kwon YK, Kang MI 2003 Outbreak of fowl cholera in Baikal teals in Korea. Avian Dis 47(4):1491-1495.

Oh YH, Moon DC, Lee YJ, Hyun BH, Lim SK 2019 Genetic and phenotypic characterization of tetracycline-resistant *Pasteurella multocida* isolated from pigs. Vet Microbiol 233:159-63.

Singh R, Blackall PJ, Remington B, Turni C 2013 Studies on the presence and persistence of *Pasteurella multocida* serovars and genotypes in fowl cholera outbreaks. Avian Pathol 42(6):581-585.

Singh R, Remington B, Blackall P, Turni C 2014 Epidemiology of fowl cholera in free range broilers. Avian Dis 58(1):124-128.

Subaaharan S, Blackall LL, Blackall PJ 2010 Development of a multi-locus sequence typing scheme for avian isolates of *Pasteurella multocida.* Vet Microbiol 141(3-4):354-361.

Townsend KM, Boyce JD, Chung JY, Frost AJ, Adler B 2001 Genetic organization of *Pasteurella multocida* cap loci and development of a multiplex capsular PCR typing system. J Clin Microbiol 39(3):924-929.

Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ 1998 Development of PCR assays for species-and type-specific identification of *Pasteurella multocida* isolates. J Clin Microbiol 36(4):1096-1100.

Wang Y, Zhu J, Lu C, Wu B, Liu D, Hang W, Liu H, Liu X 2013 Evidence of circulation of an epidemic strain of *Pasteurella multocida* in Jiangsu, China by multi-locus sequence typing (MLST). Infect Genet Evol 20:34-38.

Wilson MA, Morgan MJ, Barger GE 1993 Comparison of DNA fingerprinting and serotyping for identification of avian *Pasteurella multocida* isolates. J Clin Microbiol 31(2):255-259.

Woo YK, Kim JH 2006 Fowl cholera outbreak in domestic poultry and epidemiological properties of *Pasteurella multocida* isolate. J Microbiol 44(3):344-353.

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