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Epidemiological relationship of *Clostridium perfringens* isolated from feces and soil of horse farm using multi-locus sequence typing analysis

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ABSTRACT

We analyzed the epidemiological relationship of *Clostridium (C.) perfringens* isolated from feces and soil of horse farm using multi-locus sequence typing (MLST) and then compared it with standard strains registered in the National Center for Biotechnology Information. MLST results using MEGA 6.0 showed that total 13 clusters were formed in the phylogenetic tree of the housekeeping genes sequence of the standard strains, and the Korea Isolate Ju (KSJ) strains were classified into 8 types (cluster 4, cluster 5, cluster 6, cluster 7, cluster 8, cluster 11, cluster 12, and cluster 13). The KSJ strains were categorized into 4 groups. Each group had a high bootstrap value (>90%). These results for *C. perfringens* are considered to be helpful for performing epidemiological investigations and establishing prevention methods for diseases in the future.

Key words: *Clostridium perfringens*, horse, multi-locus sequence typing, soil.

INTRODUCTION

Clostridium (C.) perfringens is a gram-positive, anaerobic, endospore-forming, rod-shaped with a size of 0.6-2.4×1.3-19.0μm, is one of the most common foodborne pathogens in the world (Abdelrahim et al. 2019; Hussain et al. 2022) and produces at least 17 different toxins. Four major types (*alpha, beta, epsilon,* and *iota*) of toxins are produced by *C. perfringens* and classified into 5 different types (A, B, C, D, and E) as per the production of toxins (Khan et al. 2008; Deguchi et al. 2009; Diab et al. 2012; Li et al. 2013; Uzal et al. 2014; Nagahama et al. 2015; Othani and Shimizu 2016; Kiu and Lindsay 2018; Mehdizadeh Gohari et al. 2020). However, the typing system has been expanded to include toxin types F and G based on production of enterotoxin (CPE) and necrotic enteritis B-like toxin (netB), respectively (Kiu and Lindsay 2018; Rood et al. 2018).

The toxicity of *C. perfringens* depends on the toxins produced; not all *C. perfringens* are toxic, and bacterium without pathogenicity are also widely distributed in environment and easily found in gastrointestinal tract of humans and animals (Nakano et al. 2017; Mehdizadeh Gohari et al. 2021). The spores of this bacterium can survive without dying in extreme conditions of soil or sediment and human or animal feces (Li et al. 2013). Traditionally, the major toxins have provided the basis for classification of the individual strains into 5 toxin types (A-E) (Mehdizadeh Gohari et al. 2020). *C. perfringens* that are isolated from the environment or human field are mostly type A. Types B to E are mainly isolated from animals (Lee 2016). The existence of *C. perfringens* in drinking water is used as an indicator of water pollution by feces and similar pollutants (Diab et al. 2012; Uzal et al. 2014).

C. perfringens causes food poisoning in humans, and although there are differences in the amount of livestock in animals, it usually infects intestinal relationships, such as necrotic enteritis and hemorrhagic enteritis, leading to death (Li et al. 2013; Farag et al. 2023).

Microbes reside on the animal's intestine and soil; therefore, it can be contagious, especially in livestock species that grow in groups or animals that live in clusters. This can cause contraction and death of animals because of diarrhea and enteritis, resulting in economic loss (Uzal et al. 2014).

Considerable research has been conducted on *C. perfringens* at the national and international level (Yoo et al. 1997; Gkiourtzidis et al. 2001; Kim et al. 2006; Lahti et al. 2008; Deguchi et al. 2009; Xiao et al. 2012; Park et al. 2016). In particular, in humans, there are many studies on CPE toxins that cause food poisoning, and various types of toxins that cause enteritis in chickens are being studied (Lahti et al. 2008; Lee 2016). Along with studies of the toxins in these bacteria, epidemiological studies are conducted to determine the origin of the disease in cases

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of food poisoning or to identify the characteristics of the bacteria by analyzing the correlation in the diseases (Park et al. 2016).

Various epidemiologic studies using pulsed-field gel electrophoresis (PFGE) and several other molecular typing tools have been developed to investigate the epidemiological relationship among homogeneous bacteria. However, these tools are less portable due to the index of variation and difficult to compare results between laboratories (Maiden et al. 1998) and is also difficult to investigate the genetic relationship between strains isolated from different outbreaks. To overcome these hardships, multi-locus sequence typing (MLST) has emerged as a more suitable tool for large-scale global epidemiologic studies (Park et al. 2019). MLST is based on sequence comparison of internal fragments of housekeeping genes (Verma et al. 2020). The mutations of housekeeping genes are presumed to be neutral (Maiden et al. 1998), and the nucleotide changes in these genes are relatively slow; therefore, MLST is an ideal molecular tool for global epidemiological research (Enright and Spratt 1999; Jolly et al. 2018; Guerrero-Araya et al. 2021).

This study aims to analyze the epidemiological relationship of *C. perfringens* isolated from feces and soil of horse farm in South Korea using MLST and then compare it with standard strains registered in the National Center for Biotechnology Information.

MATERIALS AND METHODS

Ethical Approval: This study was carried out on the care and use of experimental animals according to the guidelines of the Animal Ethics Committee (KNU2019-0091) of Kyungpook National University in Korea.

Target strain and housekeeping genes: The present study was conducted with 20 *C. perfringens* isolated from feces of dead foals and soil of horse farm previously reported in South Korea (Park et al. 2019) and 16 standard strains (Table 1). A total of 8 housekeeping genes, including toxin genes (plc, colA), stress response genes (sodA, groEL), sigma factor sporulation (sigK), putative metabolism genes (pgK, nadA), DNA replication (gyrB) was used, and primers used in this study are shown in Table 2 (Deguchi et al. 2009; Xiao et al. 2012).

MLST Analysis: MLST analysis of *C. perfringens* was performed with 16 standard strains and 20 strains isolated in South Korea. For the MLST analysis, PCR was performed including initial denaturation for 5 min at 94°C, multiple reactions in a total of 35 cycles as follows: 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and the final extension by reacting at 72°C for 7 min. The annealing time was all equally performed at 55°C (Deguchi et al. 2009). The amplified product was analyzed for nucleotide sequence using ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, USA). The sequence of the nucleotide was arranged in the order of colA, groEL, sodA, plc, gyrB, sigK, pgK, and nadA and was aligned using the Bioedit program.

Data Analysis: The phylogenetic tree was constructed based on concatenated nucleotide sequences from 8 MLST loci to show genetic relationship between all of the *C. perfringens* sequence types using neighbor-joining (NJ) method with the bootstrap values at 1,000 replicates by MEGA 6.0 program (Tamura et al. 2013).

Table 1: Sixteen standard strains of *Clostridium perfringens* used in this study

Strain	Type	Source	Region
NCTC8239	A	Food poisoning	Europe
NCTC8533	B	Animal disease (Lamb)	Europe
NCTC8081	C	Necrotizing enterocolitis	Europe
NCTC3182	C	Animal disease (sheep)	Europe
NCTC8346	D	Animal disease (sheep)	Europe
NCTC8084	E	Animal disease (calf)	Europe
NCTC8798	A	Food poisoning	Europe
W4232	A	Food poisoning	Japan
W6205	A	Food poisoning	Japan
MR2-3	A	Healthy	Japan
M-07	A	Food isolate	Japan
M-08	A	Food isolate	Japan
T1	A	Food poisoning	Japan
T16	A	Food poisoning	Japan
VWA080	A	Food isolate	Europe
F5603	A	Sporadic diarrhea	Europe

All standard strains cited by PubMed database, National Center for Biotechnology Information, USA.

Table 2: Primers of MLST housekeeping genes used in this study

Species	Gene	Name	Sequence	Size (bp)	Analysis (bp)
Clostridium perfringens	colA	colA_F	5'-ATTAGAAAGTTTATGTACAATAGGTG-3'	816	670
		colA_R	5'-AAGACATTCTATTATTTCTATCGTAAGC-3'		
	groEL	groEL_F	5'-TACAAGATTTATTACCATTACTTGAG-3'	901	685
		groEL_R	5'-CATTTCTTTTTCTGGAATATCTGC-3'		
	sodA	sod_F	5'-CAAAAAAAGTCCATTAATGTATCCAG-3'	663	502
		sod_R	5'-TTATCTATTGTTATAATATTCTTCAC-3'		
	plc	plc_F	5'-AGGAACTCATGATTGTAACTC-3'	725	541
		plc_R	5'-GGATCATTACCCTCTGATACATCGTG-3'		
	gyrB	gyrB_F	5'-ATTGTTGATAACAGTATTGATGAAGC-3'	905	735
		gyrB_R	5'-ATTTCCTAATTTAGTTTTAGTTTGCC-3'		
	sigK	sigK_F	5'-CAATACTTATTAGAATTAGTTGGTAG-3'	643	589
		sigK_R	5'-CTAGATACATATGATCTTGATATACC-3'		
	pgk	pgk_F	5'-GACTTTAACGTTCCATTAAAAGATGG-3'	830	681
		pgk_R	5'-CTAATCCCATGAATCCTTCAGCGATG-3'		
	nadA	nadA_F	5'-ATTAGCACATTATTATCAAATTCCTG-3'	821	689
		nadA_R	5'-TTATATGCCTTTAATCTTAAATCCTC-3'		

RESULTS

The results of analysis of gene sequencing of 20 strains (KSJ) showed a new sequence type, as shown in Table 3. Of the 20 strains, KSJ-02, KSJ-22, and KSJ-23 were completely consistent with all the previously identified species profiles. However, they were identified in a new sequence type with the entire sequence of housekeeping genes. KSJ-03, KSJ-08, KSJ-09, KSJ-14, KSJ-15, KSJ-16, KSJ-25, and KSJ-28 were sequence types with 1 to 5 sequence differences that were not completely consistent with existing allelic profiles. KSJ-04, KSJ-06, KSJ-07, KSJ-11, KSJ-12, KSJ-13, KSJ-17, and KSJ-21 were identified as new nucleotide sequence types that did not match the existing allele profile.

MLST results using MEGA 6.0 showed that a total of 13 clusters were formed in the phylogenetic tree of the housekeeping genes sequence of the standard strains, and the KSJ strains were classified into eight types (cluster 4, cluster 5, cluster 6, cluster 7, cluster 8, cluster 11, cluster 12, and cluster 13), as shown in Fig. 1.

KSJ strains were categorized into four groups. Each group had a high bootstrap value (>90%). These strains were classified as follows: KSJ-14, KSJ-15, KSJ-16, KSJ-20, KSJ-25, KSJ-26, and KSJ-28 in group I; KSJ-08, KSJ-09, KSJ-11, KSJ-12, KSJ-13, KSJ-17, KSJ-22, KSJ-23, and KSJ-28 in group II; KSJ-03 in group III; and KSJ-04, KSJ-06, KSJ-07, and KSJ-21 in group IV, respectively (Fig. 2).

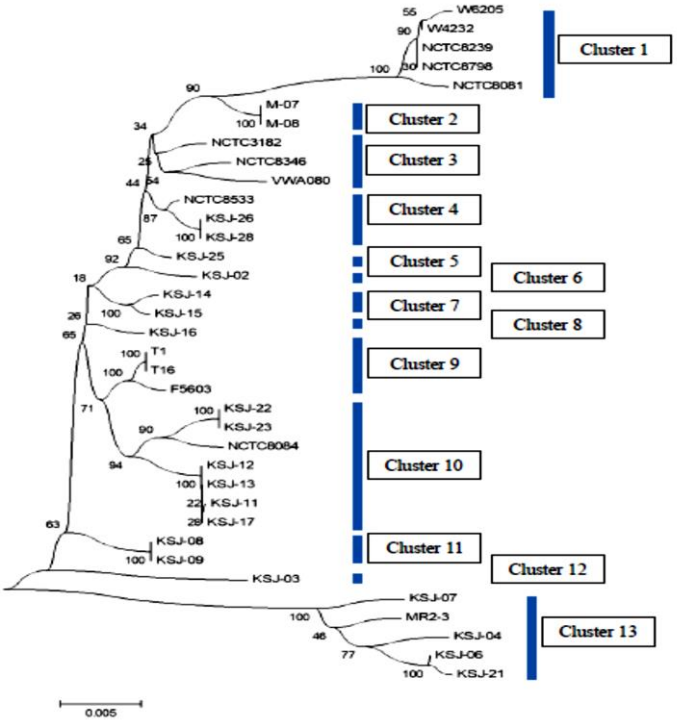

Fig. 1. Phylogenetic tree of *Clostridium perfringens* isolated from horse feces and contaminated soil in Korea with *Clostridium perfringens* standard strains. This tree was constructed by the Neighbor-Joining method, MEGA 6.0 program.

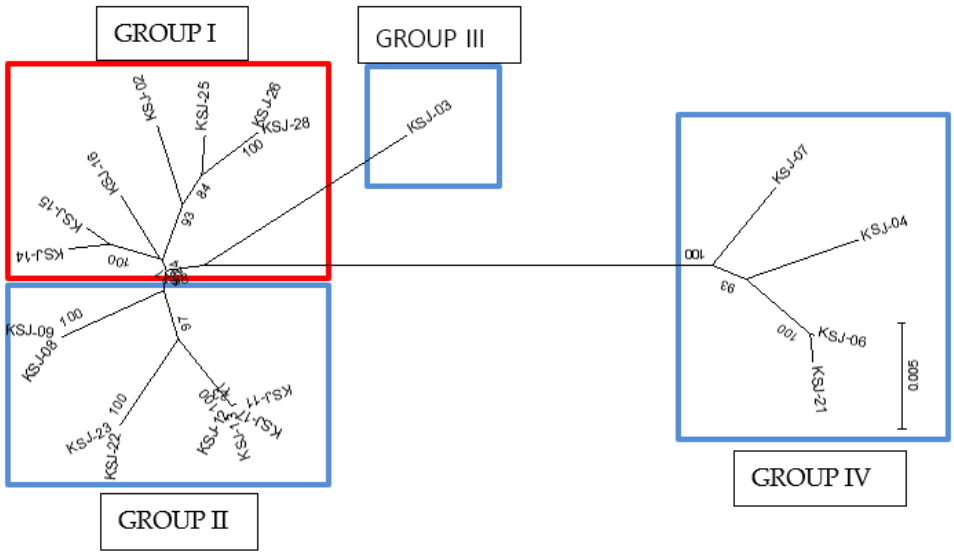

Fig. 2: Phylogenetic tree of *Clostridium perfringens* isolated from horse feces and contaminated soil in Korea. The nucleotide sequences of all 20 new strains (KSJ strains) are separated 4 groups. Red circle included foal death occurred region (KSJ-15 and KSJ-25, Jeju-Do). This tree was constructed by the Neighbor-Joining method, MEGA 6.0 program.

Table 3: *Clostridium perfringens* isolates sequence types and region

Sr. No.	Strains	Alleles	Sequence type	Region
1	KSJ-02	6-3-1-1-4-3-5-19	New	Chungcheong-Do
2	KSJ-03	12*-33-28*-36-18-34*-25-38	New	Kyungki-Do
3	KSJ-04	18*-18-13*-N-11*-N-14*-N	New	Kyungki-Do
4	KSJ-06	24*-18*-13*-N-11-N-14*-N	New	Kyungki-Do
5	KSJ-07	18*-18*-3-N-8-N-N-N	New	Chonra-Do
6	KSJ-08	32*-29*-1*-8*-3-31*-4*-1*	New	Chonra-Do
7	KSJ-09	32*-29*-1*-8*-3-31*-4*-1*	New	Chonra-Do
8	KSJ-11	7*-6*-15*-N-7-6-2*-1	New	Chonra-Do
9	KSJ-12	7*-6*-15*-N-7-6-2*-1	New	Chonra-Do
10	KSJ-13	7*-6*-15*-N-7-6-2*-1	New	Chonra-Do
11	KSJ-14	7*-4*-3-11-4-1*-5-1	New	Chonra-Do
12	KSJ-15	3-19-1-11*-4-4-5-1	New	Jeju-Do
13	KSJ-16	26*-3-1-1-4-10*-2*-1	New	Jeju-Do
14	KSJ-17	7*-6*-15*-N-7-6-2*-1	New	Jeju-Do
15	KSJ-21	5*-18*-13*-N-11-N-14*-N	New	Gyeongsang-Do
16	KSJ-22	6-5-24-19-7-33-4-1	New	Gyeongsang-Do
17	KSJ-23	6-5-24-19-7-33-4-1	New	Gyeongsang-Do
18	KSJ-25[a]	4-1-3-1-1-4-5-20*	New	Jeju-Do
19	KSJ-26	6*-1*-3-13-1-1-2-5	New	Gyeongsang-Do
20	KSJ-28	6*-1*-3-13-1-1-2-5	New	Gyeongsang-Do

N: new allelic sequences; *: 1 to 5 allelic base sequences are not 100% matched with pre study (Xiao et al. 2012) and *C. perfringens* MLST homepage (https://pubmlst.org/cperfringens; Jolley et al. 2018).

DISCUSSION

The MLST assay can distinguish even homogeneous strains that are closely related via various allele combinations (Spratt 1999). In addition, by determining the sequence of each allele and then determining the sequence type, phylogenetic classification between the strains is possible (Maiden et al. 1998). This method is particularly widely used in the field of medicine to epidemiologically distinguish between pathogenic and non-pathogenic strains of homogeneous bacteria (Park et al. 2019). In the case of *C. perfringens*, there are many genetic variations within the same strain (Deguchi et al. 2009), insufficient research results, and many difficulties in using internationally recognized MLST databases.

Conventional domestic and overseas epidemiological studies of *C. perfringens* have focused on the existence of enterotoxin gene (CPE) that is primarily designated as a major source of food poisoning in humans, or whether the gene's location is chromosome or plasmid, and whether there is a correlation with disease according to the location of CPE gene (Lahti et al. 2008; Deguchi et al. 2009; Xiao et al. 2012). However, MLST analysis of *C. perfringens* is not performed much in animals. Although there is a previous study of the correlation between toxins and diseases or between strains isolated from humans and animals (Jost et al. 2006; Chalmers et al. 2008), limited data are available on the phylogenetic classification or animal-specific analysis compared to studies performed in humans. Therefore, more research is needed, focusing on bacteria isolated from animals.

A total of 13 clusters were generated via an epidemiological analysis between standard strains and 20 isolates, and no mechanical correlation with the type was shown, as in previous studies. However, a phylogenetic tree that was distinguished from each other as per the presence or absence of CPE or the location of the CPE gene appeared (Lahti et al. 2008).

The strains isolated from Korea were found in eight clusters, of which cluster 4, cluster 10, and cluster 13 formed the same cluster as the standard strains. Cluster 4 and cluster 10 both formed clusters, such as standard strains (NCTC8533 and NCTC8084) isolated from animals, and the bootstrap value (%) was high [cluster 4 (87%), cluster 10 (90%)].

It is highly likely that the strains of animal origin are common ancestors. Since both NCTC8533 and NCTC8084 are strains isolated from Europe, it is estimated that the domestic isolates in the same cluster also originated in Europe. This appears to be closely related to European strains systematically because most domestic horses, except Halla horses (native crossbreed) are thoroughbreds imported from another country.

In the case of cluster 13, the bootstrap value with the standard strain MR2-3 was low (46%); therefore, it is difficult to say that it has a common ancestor; however, in the case of MR2-3, they are bacteria isolated from a healthy person, and domestic isolates forming the same group also have a genetic correlation that is isolated, irrespective of the disease. In particular, results analysis of a prior study (Diab et al. 2012), MR2-3 existed alone because of its low genetic association with other *C. perfringens*; however, the four strains isolated in this study (KSJ-04, KSJ-06, KSJ-07, KSJ-21) did not have a common ancestor, but showed high genetic relevance; thus, the cluster was formed by several strains rather than alone.

As per an analysis of the epidemiological relationship between domestic isolates, *C. perfringens* was classified into four groups. The divided groups did not show any difference based on the location where the *C. perfringens* were isolated, such as inland or Jeju Island in South Korea. Moreover, there was no difference as per type in

comparison with the standard strains. However, group 4 had a lower systematic relationship than the other three groups. This is analyzed with the same results as cluster 13 shown in the comparison and analysis with the standard strains.

In addition, the bacteria (KSJ-15, KSJ-25) isolated from the place where foal dead took place appeared together in group I. KSJ-15 and KSJ-25 belong to *C. perfringens* type C with β-toxin (CPB), and type C causes intestinal toxemia in infected foals, causing acute death within 1–2 d of birth (Diab et al. 2012). The *C. perfringens* type C isolated in Korea is characterized in that all strains except KSJ-03, which form a group alone, are formed in the same group I.

In conclusion, we attempted to analyze the epidemiological relationship centering on the strains isolated from the feces of horses and from contaminated soil in South Korea. We confirmed that the domestic isolates of human beings and animals had an epidemiologic correlation, that is, there was no regional genetic relationship between the strains. However, we found a close genetic relationship between the strains causing the disease. In addition, we confirmed that it is systematically close to the standard strains isolated from Europe (Camargo et al. 2022).

In this study, MLST analysis was performed using strains that were isolated from domestic horses, and various genetic relationships were derived; however, these findings are limited to horses. *C. perfringens* is a representative bacterium that causes diseases not only in humans, but also in animals. Thus, it is necessary to derive MLST results for each species, such as cattle, pigs, and chickens in the future to compare the genetic characteristics of pathogenic strains. Research focusing on such phylogenetics is considered useful for performing epidemiological investigations or establishing prevention methods for diseases in the future.

Conclusions: MLST of *C. perfringens* results using MEGA 6.0 showed that total 13 clusters were formed in the phylogenetic tree of the housekeeping genes sequence of the standard strains, and the Korea Isolate Ju (KSJ) strains were classified into eight types (cluster 4, cluster 5, cluster 6, cluster 7, cluster 8, cluster 11, cluster 12, and cluster 13). The KSJ strains were categorized into four groups. Each group had a high bootstrap value (>90%). These results for *C. perfringens* are considered to be helpful for performing epidemiological investigations and establishing prevention methods for diseases in the future.

Author's Contribution: All research protocols and animal experiments in this study were designed, and conducted by CS Park, who also contributed to data acquisition. GJ Cho contributed to the interpretation of the experimental results and the writing the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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