Genetic Diversity of *Clostridium perfringens* Strains Isolated from Broiler Chickens Revealed by PFGE Analysis in China and Pakistan

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**ABSTRACT**

*Clostridium perfringens* (*C. perfringens*) is widely distributed in broiler chickens causing clinical and subclinical enteritis and is especially known for causing necrotic enteritis (NE). There are numerous reports of NE outbreaks in Pakistan as well as China but there is a lack of information related to PFGE profile from both the countries. To close this gap, we designed this study and obtained samples from broiler chicken farms located in 3 different regions of Pakistan and 4 different regions of China. A total of 79 fecal swabs (Pakistan=29; China=50) were collected and grown on FTA media. Further, isolates were grown on TSE agar and agar colonies were selected for DNA extraction. All 79 isolates were tested for toxin profiles by PCR (α-gene; beta-2; netB gene) and PFGE profiling (pulsotypes analysis). Toxinotyping results revealed that all the isolates (n=50) from China were type A (α-toxin positive) while 23 and 6 isolates (n=29) from Pakistan were type A (α-toxin positive) and type G (α-toxin, NetB positive), respectively. Toxinotyping revealed α-toxin is highly prevalent in both the countries while from Pakistani isolates, NetB toxin was also detected. PFGE discriminated 79 isolates into 45 different PFGE patterns (pulsotypes). The analysis further showed different pulsotypes originating from China and Pakistan and isolates were subtyped by *Smal*. The results showed high genetic polymorphism in *C. perfringens* even within the same strain. These preliminary findings of genetic variations will further help to design control strategies.

**INTRODUCTION**

*Clostridium perfringens* (*C. perfringens*) is an anaerobic spore-forming Gram-positive bacterium (*Xiu et al., 2020*) responsible for necrotic enteritis (NE) in poultry industry with an estimated loss between 2$ and 6$ billion USD globally (*Mwangi et al., 2019*). Predisposing ages for this disease ranges 2 to 6 weeks in either clinical or subclinical form (*Skinner et al., 2010*). In the recently revised toxinotyping scheme of *C. perfringens*, it was classified into seven types (A to G) (Table 2) in addition to *C. perfringens* enterotoxin (CPE) and NetB (*Rood et al., 2018*). The pathogenic clostridial species can be classified into three groups, based on their toxin activity (enterotoxic, histotoxic, neurotoxic) on the target tissues (*Rood et al., 1997*) while *C. perfringens* is the most frequently isolated clostridial species around the globe (*Li et al., 2013*). In NetB positive *C. perfringens* poultry strains, genetics provides a crucial role in necrotic enteritis (NE) pathogenicity by altering plasmid maintenance, carbohydrate metabolism, and iron acquisition (*Lepp et al., 2013*). The prevalence of NE in poultry birds is often related to predisposing factors like increased intestinal contents’ viscosity, unbalanced ration composition, and co-occurrence of other pathogens like different species of *Eimeria* (*Rodgers et al., 2014*). Understanding *C. perfringens* pathogenesis is essential to prevent and control NE outbreaks (*Allaart et al., 2013*).

With regard to the epidemiological surveillance point of view and describing bacterial genotypic diversity, the pulsed-field gel electrophoresis (PFGE) is the “gold standard” and third-generation molecular typing tool for

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comparing and analyzing DNA fingerprinting patterns of *C. perfringens* strains (Nassonova, 2008; Goering, 2010). At present PFGE is considered to be the primary method in outbreak investigation, disease surveillance, and disease clustering programs. In molecular epidemiological studies, where economical, simple, and less laborious methods are required to screen a huge number of samples, PFGE stands the best practicable technique (Lopez-Canovas et al., 2019).

As far as we know, there is no report related to molecular typing of *C. perfringens* type A and *C. perfringens* type G isolates through PFGE from different geographical regions of China and Pakistan. The current study was designed to appraise the genetic relatedness of *C. perfringens* type A and type G isolates successfully recovered from broiler chicken using PFGE to explain the variation in *C. perfringens* subtypes in broiler birds.

**MATERIALS AND METHODS**

**Sample collection:** Sampling was carried out from commercial broiler poultry farms located in different provinces of China and Pakistan (Fig. 2). The farms included in this study had a complaint of intestinal ailments and blood in droppings of poultry birds. A total of 79 fecal swabs were taken from broiler chickens. Among them, 50 samples were collected from Hainan, Gansu, and Yunnan from China, and 29 samples were collected from South Punjab and Punjab of Pakistan (Table 1).

**Isolation of bacterial strains:** Fecal swabs were inoculated into 5 ml thioglycollate (FTA) broth and incubated at 37°C (Don Whitely DG-250 anaerobic workstation, United Kingdom) for 24h. Subsequently, 100μl of pre-enriched FTA broth was spread on tryptose sulphite cycloserine agar base enriched with 7% egg yolk and supplemented with D-cycloserine (Solarbio, Beijing, China). A black colony harboring a positive lecithinase action was selected and cultured (Fig. 1). For identification and purity checks, *C. perfringens*, were streaked on Columbia blood agar (Huan Kai Microbial (HKM) Sci & Tech, Guangzhou, China) containing 5% defibrinated sheep blood and evaluated for typical double zone hemolysis associated with *C. perfringens*. Isolates were preserved in 50% glycerol at -80°C till further use.

**Extraction of bacterial DNA:** Genomic DNA of *C. perfringens* was extracted from overnight FTA broth culture inoculated with a single colony by Ultraclean Microbial DNA Isolation Kit (MoBio, Germantown, Maryland, USA) rendering to the manufacturer’s directives with minor modification to attain high concentration. To ensure the quality assertion and purity of extracted DNA was determined by NanoDrop™ 2000 (Thermo Scientific Inc. Waltham, MA, USA). The DNA was stored at -20°C for further genotyping analysis.

**16S rRNA gene amplification:** All 79 strains of *C. perfringens* were further confirmed by the species-specific primer of 16s rRNA gene amplification by PCR (Kikuchi et al., 2002).

**Toxinotyping of isolated strains:** Genes encoding toxin proteins including plc, cph, etx, cpe, netB, and cphb2 were detected by PCR with slight modifications (Chalmers et al., 2008; Keyburn et al., 2008). American Type Culture Collection ATCC-3624 (toxin type A, α-toxin positive) and China Institute of Veterinary Drug Control, Beijing, China including CVCC-54 (toxin type B, α-, β- and ε toxin positive), CVCC-61 (toxin type C, α-, and β toxin positive) and CVCC-81 (toxin type D, α- and ε toxin positive) were utilized as standard strains for toxinotyping (A, B, C, and D, respectively) and as positive controls for cphb2 and cpe. PCR reactions were performed with an initial denaturation at 96°C for 5 min, followed by 35 cycles at 96°C for 1min, 55°C for 1min, and 72°C for 50s and a final extension at 72°C for 10 min. For NetB, the assay conditions were modified as follows: initial denaturation at 94°C for 3 m; 35 cycles at 94°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 5 min. Ethidium bromide (10mg/ml) by (GenStar, Beijing, China) was used to stained 1.2% agarose gel of amplified products. PCR amplified products on the gels were extracted and purified by EZNA® Gel Extraction Kit (Omega Bio-Tek, USA) and sequenced (Tsingke Biotechnology Company Xian, China) to ensure the identity with reference sequences.

**Pulsed-Field Gel Electrophoresis (PFGE) typing of *C. perfringens* strains:** Genotyping of *C. perfringens* was carried out by following the standard protocol of PFGE. All necessary steps like e.g. plug preparation, restriction digestion, and electrophoresis running conditions according to assay as explained earlier (Chalmers et al., 2008). The Smal (New Bio, England) restriction enzyme was used at appropriate conditions recommended by the manufacturer. Pulse Field Certified Agarose 1% (Bio-Rad, USA) was used to segregate the Restriction fragments in Tris-borate solution and EDTA (TBE buffer, Solarbio, Beijing, China). The gel running time assigned 18 h, voltage of 6 V/cm, and a linearly ramped pulse time of 0.5 to 38s. The CHEF-DR III system (Bio-Rad, USA) was used to separate the macro restriction fragments in 1% agarose gel in 0.5×TBE buffer and gel stained with ethidium bromide (10mg/ml) by (GenStar, Beijing, China) followed by a destaining step in water for 20 min and the image was captured by a ChemiDoc CRX® Image analyzer (Bio-Rad USA) as tiff files.

**Computational analysis of PFGE patterns:** An analysis of the patterns obtained from the restrictive Smal endonuclease was carried out with PFGE standards and analyzed using the software package (BioNumerics version 7.6, Applied Maths, Inc., Austin, TX, USA). The comparison was based on a band (line) evaluation for each type and the similarity analysis was performed using the Dice coefficients (S0) with a custom tolerance of 1.5%. The sort of dendrogram was made by unweighted pair group impressions formed by the unweighted pair group method with arithmetic mean (UPGMA).
Further (1995–)
activity from both the China and Pakistan
D73
D72
D71
D70
D69
D67
D65
D64
D63
D62
D60
D59
D57
D52
D50
D43
D37
D36
D35
D28
D27
D23
D22
D20
D17
D08
D04
D01

Fig. 1: Black colonies of C. perfringens on Tryptose Sulphite Cytochrome
(TSC) agar medium.

Electrophoresis Pattern (EP) optimization and optimal enzyme activity: 79 isolates with optimal enzymes Smal were analyzed and digestion resulted in a band pattern (line) due to the cutting of bacterial DNA restriction sites. The Simpson diversity index (S0 value) was used to evaluate the discriminatory power (Hunter and Gaston, 1988).

RESULTS

Isolate identification: All 79 isolates were detected as C. perfringens through culture, and 16S rRNA gene amplification by PCR followed by sequences analysis. The representative sequences can be accessed at NCBI (accession number: MN365133-MN365150).

Toxinotyping and confirmation of genes encoding toxin proteins by PCR: The toxontypes of 79 isolated C. perfringens strains were confirmed by toxins specific PCR. The results revealed that the 79 isolated C. perfringens strains belong to type A and type G. From China, all the isolates 50 out of 50 (100%) were type A while 23 out of 29 (79%) and 6 out of 29 (21%) were type A and type G, from Pakistan respectively (Table 2).

Pulsed field gel electrophoresis: We analyzed 79 C. perfringens strains and provided reasonable patterns with an endonuclease, Smal digestion led to 45 different pulsotypes which showed higher discriminatory power of Smal digestion activity and had the ability to type all C. perfringens strains and achieved the satisfactory typeability of 100% (Fig. 3). We analyzed the result three times of candidate isolates from multiples gel runs for conformity of banding pattern ascertainning satisfactory reproducibility by PFGE protocol.

Interpretation of PFGE: Based on PFGE profiles, obtained patterns showed high polymorphism on the basis endonucleases enzyme Smal activity from both the countries which is widely used to interpret PFGE band patterns (Tenover et al., 1995). Strains with 2-3 band differences were considered to be closely related while strains with 4-6 band differences were regarded as possibly related. Strains with 7 or more band differences were thought to be unrelated. In our study, we also confirmed with integrity due to closely related interpretation patterns by Tenover et al. (1995). Further clustering on the basis of the band pattern was done.

Table 1: Sampling details of C. perfringens from broiler chickens of China and Pakistan

<table>
<thead>
<tr>
<th>Numbers</th>
<th>Strains</th>
<th>Year</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>D01</td>
<td>Cp-Ch-TNAN-201401</td>
<td>2014</td>
<td>YunNan</td>
</tr>
<tr>
<td>D02</td>
<td>Cp-Ch-TNAN-201402</td>
<td>2014</td>
<td>YunNan</td>
</tr>
<tr>
<td>D03</td>
<td>Cp-Ch-TNAN-201403</td>
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<tr>
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<tr>
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<td>Cp-Ch-TNAN-201405</td>
<td>2014</td>
<td>YunNan</td>
</tr>
<tr>
<td>D06</td>
<td>Cp-Ch-TNAN-201406</td>
<td>2014</td>
<td>YunNan</td>
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<tr>
<td>D07</td>
<td>Cp-Ch-TNAN-201407</td>
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<td>D08</td>
<td>Cp-Ch-TNAN-201408</td>
<td>2014</td>
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<tr>
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<td>Cp-Ch-TNAN-201411</td>
<td>2014</td>
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</tr>
<tr>
<td>D12</td>
<td>Cp-Ch-TNAN-201412</td>
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**Table 2:** New revised classification (2018) of *C. perfringens* typing scheme toxin-based

<table>
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<tr>
<th>Toxin produced</th>
<th>α-toxin</th>
<th>β-toxin</th>
<th>ε-toxin</th>
<th>i-toxin</th>
<th>CPE</th>
<th>NetB</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
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**Clustering analysis:** Dendrogram of *C. perfringens* strains was confined with *SmaI* and lambda reference ladder (New Bio, England). The extent of the main DNA piece of *C. perfringens* was assessed by modifying the electrophoresis settings. Considering the adopted UPGMA parameters, the isolates were grouped in five (A-G) main clusters with similarities ranging from 60 to 95% (BioNumerics 7.6, Austin, TX, USA) and generating 45 pulsotypes on band pattern basis (Fig. 4).

**Clostridium perfringens clustering analysis:** Clustering analysis on the basis of band (line) pattern revealed that the isolates from South Punjab, Punjab, and Yunnan fall in cluster-A with the same pulsotypes. Cluster-B has similarity among the strains from Gansu, Yunnan, Punjab, and Hainan which lies in the same cluster but different pulsotypes. Group-C, the genetic profile of Hainan and Yunnan were similar and cluster-D consisted of Yunnan, Punjab, and South Punjab showing similar genetic patterns. Cluster-E is more diverse both genotypically and phenotypically because it shares samples from all three provinces of China and Pakistan. Apart from this, Cluster-B (1), -D (1), and -E (4) contain type G strains and cluster E is richer regarding genomic diversity as mentioned above. The value of Simpson Index with 45 pulsotypes was found to be D-value of 0.98.

**DISCUSSION**

Clostridial NE remains to pose encounters for the poultry industry globally. The new classification of the *C. perfringens* strains isolated from broiler flocks included in this study, *C. perfringens* was confirmed in all the samples used in this study. These results can be explained by the fact that *C. perfringens* is a commensal bacterium found in the gastro-intestinal tract of animals and humans (Uzal et al., 2018).

In the present study, the isolates were *C. perfringens* type A and type G (Table 2) but were found negative for *cpe* showing coherence with prior studies (Gaucher et al., 2015). The *plc* toxin of *C. perfringens* has been reported to be the key virulence factor in NE pathogenesis (Fukata et al., 1988). In this study, all isolates of *C. perfringens* from intestinal contents carried the *plc* gene, indicating that all of them belonged to toxinotype A. Type A strains produce α-toxin, and the VirR/VirS system is responsible for the synthesis of α-toxin (Shimizu et al., 2002). Production and regulation of α-toxin was thought to be a decisive cause for *C. perfringens* type A pathogenicity but later on, many experimental results revealed that α-toxin is not the major cause for NE in poultry (Gholamiandekhordi et al., 2006) and results of the current study are in conformity with the latest findings.
Fig. 4: Schematic representation (genetic profile, isolate identification, toxinootypes, serotype,) of 79 Clostridium perfringens isolates obtained from the poultry birds of China and Pakistan. Macro-restriction pattern was conducted with SmaI. Dendrogram obtained using BioNumerics 7.6 software and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Dice (Opt: 1.0%) (Tol: 1.0–1.5%).
PFGE (Gholamiandekhordi et al., 2006) isolates from China and Pakistan poultry flocks were of toxin type A and type G. In this study, a number of isolates harbored cpb2 gene. PCR based detection of the gene encoding the β-2-toxin was successfully done in sixteen samples (20%) of the broiler isolates associated with C. perfringens. Huge genetic diversity was found between isolates obtained from different poultry flocks due to different geographical regions.

A recent study found that NetB alone was unable to restore the full virulence of C. perfringens (Zhou et al., 2017). The outcomes of studies indicate that additional genes are involved in the regulation of NEloc-1 for pathogenicity and trigger the regulation of NetB to cause disease. Besides, NetB is highly influential regarding its activation on environmental factors (Parreira et al., 2016). NetB toxin also shows a strong relation with predisposing factor environmental factors like stress, feeding formulation, etc. for its expression, to cause clinical infection. In this study, it was observed that C. perfringens isolates from a chicken suffering from NE were highly clonal. The genetic relatedness amongst NE positive and NE negative broilers was fairly low. There were diverse genotypes of C. perfringens in different isolates. The isolates from broilers (n=6) affected by NE also shared genetic relatedness with different genotypes ascertained among them. Previous studies have indicated that C. perfringens isolates from NE outbreaks with high mortality rates in the flock had a close genetic relatedness while C. perfringens isolates from healthy birds have a low genetic relatedness (Gholamiandekhordi et al., 2006).

For PFGE characterization, C. perfringens strains were typed successfully. PFGE analysis of isolates showed a wide genetic variation on the basis of band pattern. The clustering analysis by PFGE data depicted the highest degree discrimination due to 45 pulsortypes. Isolates from different topographic regions constituted five clades (A-E) of the dendrogram. PFGE clearly established a clonal relationship between related strains. Although, there were a few impertinent isolates with numerous fragments that transmigrated, similar to those reported previously (Canard and Cole, 1989).

The results showed wide genetic variation diversity and no distinct relationship association between the origins of isolates and the PFGE pattern was found. On the basis of band similarity, many of the isolates clustered together in the dendrogram. In this study, none of the isolates was degraded. Therefore, PFGE can be declared as an appropriate technique for epidemiological investigation for gut-related diseases in poultry induced by C. perfringens.

Our investigation demonstrated that PFGE patterns from two countries (China and Pakistan) provided the epidemiological data that can be expanded with collaboration for better understanding. Pulse Net (Martin et al., 2006) Europe recommends settings of both optimization and position tolerance at 1.5% for band comparison in accordance with Pulse net USA. Although complying with these settings, strains visually indistinguishable may still be considered different according to the clustering analysis (Hamdi et al., 2007). Nevertheless, to improve the correct interpretation of subtyping data, the availability of a large and diverse PFGE type database is needed.

Conclusions: In summary, the present study provides insights into genetic diversity of C. perfringens in China and Pakistan. PFGE is an important tool in modern genomics, as it allows the separation of chromosomal DNA and their significant fragments and provides significant information regarding genomic profiles. This is the first report from China and Pakistan and this study will help to understand the disease pattern on the basis of toxin and PFGE profiles.

Data availability: The data (accession number: MN365133-MN365150) provoked in this study can be found in the GenBank sequence database (https://www.ncbi.nlm.nih.gov/genbank/).

Authors contribution: MUZ Khan conducts most of the experimental work and drafted the manuscript. BH Liu contributes to data analysis. SL Yang has supported to the reviewing and editing of the manuscript. X. Xu and YH Wang sample and isolate Clostridium perfringens strains from China chicken farms. JP Cai develops the idea, design the outline, and critically revised the draft. All authors edited, read, and approved the final version of the manuscript.

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