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RESEARCH ARTICLE

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

Muhammad Umar Zafar Khan[1,2], Baohong Liu [1,2] Shunli Yang[1,2], Xiao Xu[1,2], Yanhua Wang[1, 2] and Jianping Cai[1,2]*

[1]State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China. 730046; [2]Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Disease and Zoonoses, Yangzhou University, Yangzhou 225009, PR China
*Corresponding author: caijianping@caas.cn

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A B S T R A C T

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 black 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 *SmaI*. 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.

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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 the gel electrophoresis (PFGE) is the "gold standard" and third-generation molecular typing tool for

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℃ (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 reaction 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℃ till further use.

Extraction of bacterial DNA: Genomic DNA of *C. perfringens* was extracted from overnight FTA broth culture inoculated with a single colony plate 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℃ 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*, *cpb*, *etx*, *cpe*, *netB,* and *cpb2* 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 *cpb2* and *cpe*. PCR reactions were performed with an initial denaturation at 96℃ for 5 min, followed by 35 cycles at 96℃ for 1min, 55℃ for 1min, and 72℃ for 50s and a final extension at 72℃ for 10 min. For NetB, the assay conditions were modified as follows: initial denaturation at 94℃ for 3 m; 35 cycles at 94℃ for 30s, 48℃ for 30 s and 72℃ for 30s; and a final extension at 72℃ 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 *SmaI* (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) (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 *Sma*I 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 (S_D) 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).

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

Numbers	Strains	Year	Area
D01	Cp-Ch-YNAN-201401	2014	YunNan
D02	Cp-Ch-YNAN-201402	2014	YunNan
D03	Cp-Ch-YNLV-201401	2014	YunNan
D04	Cp-Ch-YNML-201701	2017	YunNan
D05	Cp-Ch-YNSL-201701	2017	YunNan
D06	Cp-Ch-YNSL-201702	2017	YunNan
D07	Cp-Ch-YNML-201702	2017	YunNan
D08	Cp-Ch-GNSL-201601	2016	GanSu
D09	Cp-Ch-YNSL-201602	2016	YunNan
D10	Cp-Ch-YNSL-201401	2014	YunNan
D11	Cp-Ch-GSJT-201501	2015	GanSu
D12	Cp-Ch-YNLV-201402	2014	YunNan
D13	Cp-Ch-YNLV-201403	2014	YunNan
D14	Cp-Ch-YNJT-201401	2014	YunNan
D15	Cp-Ch-YNSL-201402	2014	YunNan
D16	Cp-Ch-YNSL-201403	2014	YunNan
D17	Cp-Ch-HNLV-201404	2014	HaiNan
D18	Cp-Ch-HNSL-201404	2014	HaiNan
D19	Cp-Ch-HNSL-201405	2014	HaiNan
D20	Cp-Ch-HNQH-201501	2015	HaiNan
D21	Cp-Ch-YNQH-201502	2015	YunNan
D22	Cp-Ch-HNQH-201503	2015	HaiNan
D23	Cp-Ch-YNQH-201504	2015	YunNan
D24	Cp-Ch-YNLV-201405	2014	YunNan
D25	Cp-Ch-YNQH-201505	2015	YunNan
D26	Cp-Ch-YNSL-201406	2014	YunNan
D27	Cp-Ch-GNSL-201407	2014	GanSu
D28	Cp-Ch-GSJT-201502	2014	GanSu
D29	Cp-Ch-YNLV-201406	2014	YunNan
D30	Cp-Ch-YNSL-201408	2015	YunNan
D31	Cp-Ch-YNJT-201503	2015	YunNan
D32	Cp-Ch-YNLV201405	2014	YunNan
D33	Cp-Ch-HNLV-201407	2014	HiaNan
D34	Cp-Ch-HNLV-201408	2014	HaiNan
D35	Cp-Ch-YNLV-201409	2014	YunNan
D36	Cp-Ch-YNWC-201501	2015	YunNan
D37	Cp-Ch-GNWC-201502	2015	GanSu
D38	Cp-Ch-HNLV-201410	2014	HaiNan
D39	Cp-Ch-YNSL-201409	2014	YunNan
D40	Cp-Ch-YNJT-201504	2015	YunNan
D41	Cp-Ch-YNQH-201506	2015	YunNan
D42	Cp-Ch-YNLV-201411	2014	YunNan
D43	Cp-Ch-YNLV-201412	2014	YunNan
D44	Cp-Ch-YNLV-201413	2014	YunNan
D45	Cp-Ch-YNSL-201410	2014	YunNan
D46	Cp-Ch-YNSL-201411	2014	YunNan
D47	Cp-Ch-YNSL-201412	2014	YunNan
D48	Cp-Ch-YNLV-201414	2014	YunNan
D49	Cp-Ch-YNSL-201413	2014	YunNan
D50	Cp-Ch-YNLV-201801	2017	YunNan
D51	Cp-Pk-PNJB-201801	2018	Punjab
D52	Cp-Pk-PNJB-201802	2018	Punjab
D53	Cp-Pk-PNJB-201803	2018	Punjab
D54	Cp-Pk-PNJB-201804	2018	Punjab
D55	Cp-Pk-PNJB-201805	2018	Punjab
D56	Cp-Pk-PNJB-201806	2018	Punjab
D57	Cp-Pk-PNJB-201807	2018	Punjab
D58	Cp-Pk-PNJB-201808	2018	Punjab
D59	Cp-Pk-PNJB-201819	2018	Punjab
D60	Cp-Pk-PNJB-201810	2018	Punjab
D61	Cp-Pk-SPNJB-201811	2018	Punjab
D62	Cp-Pk-SPNJB-201812	2018	S Punjab
D63	Cp-Pk-SPNJB-201813	2018	S Punjab
D64	Cp-Pk-SPNJB-201814	2018	S Punjab
D65	Cp-Pk-SPNJB-201815	2018	S Punjab
D66	Cp-Pk-SPNJB-201816	2018	S Punjab
D67	Cp-Pk-SPNJB-201817	2018	S Punjab
D68	Cp-Pk-PNJB-201818	2018	Punjab
D69	Cp-Pk-SPNJB-201819	2018	S Punjab
D70	Cp-Pk-SPNJB-201820	2018	S Punjab
D71	Cp-Pk-SPNJB-201821	2018	S Punjab
D72	Cp-Pk-SPNJB-201822	2018	S Punjab
D73	Cp-Pk-SPNJB-201823	2018	S Punjab
D74	Cp-Pk-SPNJB-201824	2018	S Punjab
D75	Cp-Pk-SPNJB-201825	2018	S Punjab
D76	Cp-Pk-PNJB-201826	2018	S Punjab
D77	Cp-Pk-PNJB-201827	2018	S Punjab
D78	Cp-Pk-PNJB-201828	2018	Punjab
D79	Cp-Pk-PNJB-201829	2018	Punjab

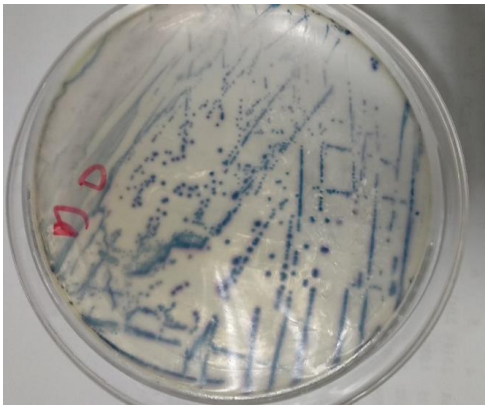

Fig. 1: Black colonies of *C. perfringens* on Tryptose Sulphite Cycloserine (TSC) agar medium.

Electrophoresis Pattern (EP) optimization and optimal enzyme activity: 79 isolates with optimal enzymes *SmaI* were analyzed and digestion resulted in a band pattern (line) due to the cutting of bacterial DNA restriction sites. The Simpson diversity index (S_D 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 toxinotypes 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, *SmaI* digestion led to 45 different pulsotypes which showed higher discriminatory power of *SmaI* 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 ascertaining satisfactory reproducibility by PFGE protocol.

Interpretation of PFGE: Based on PFGE profiles, obtained patterns showed high polymorphism on the basis endonucleases enzyme *Sma*I 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.

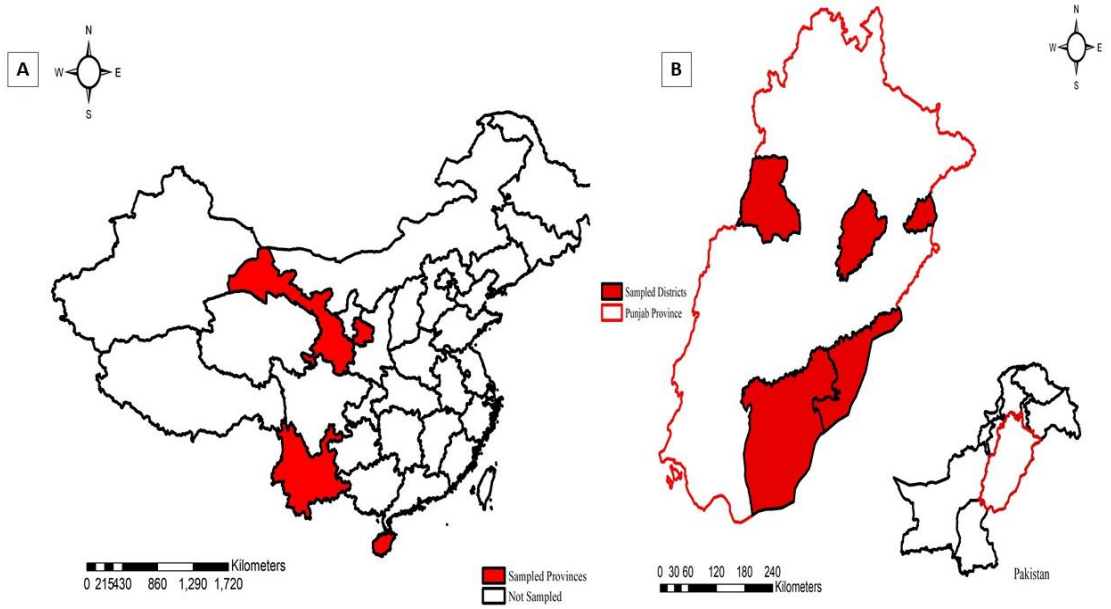

Fig. 2: Map showing sampling sites of broiler Chicken from different Provinces of China (A) and Pakistan (B) generated by ArcGIS10.2.2 software (Esri, Redlands, CA, USA).

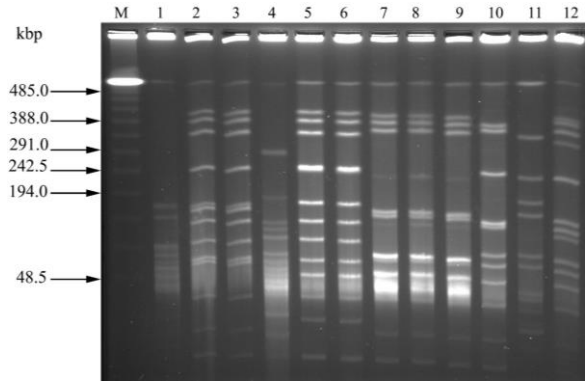

Fig. 3: Pattern obtained by PFGE *SmaI*, Marker = (PFGE Lambda Marker New England Bio, UK) lanes: 1-12 strains of *C. perfringens* of broiler chicken showing different DNA fingerprinting pattern.

Table 2: New revised classification (2018) of *C. perfringens* typing scheme toxin-based

	Toxinotypes					
Toxin produced	α-toxin	β-toxin	ε-toxin	i -toxin	CPE	NetB
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/-	-
D	+	-	+	-	+/-	-
E	+	-	-	+	+/-	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

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.

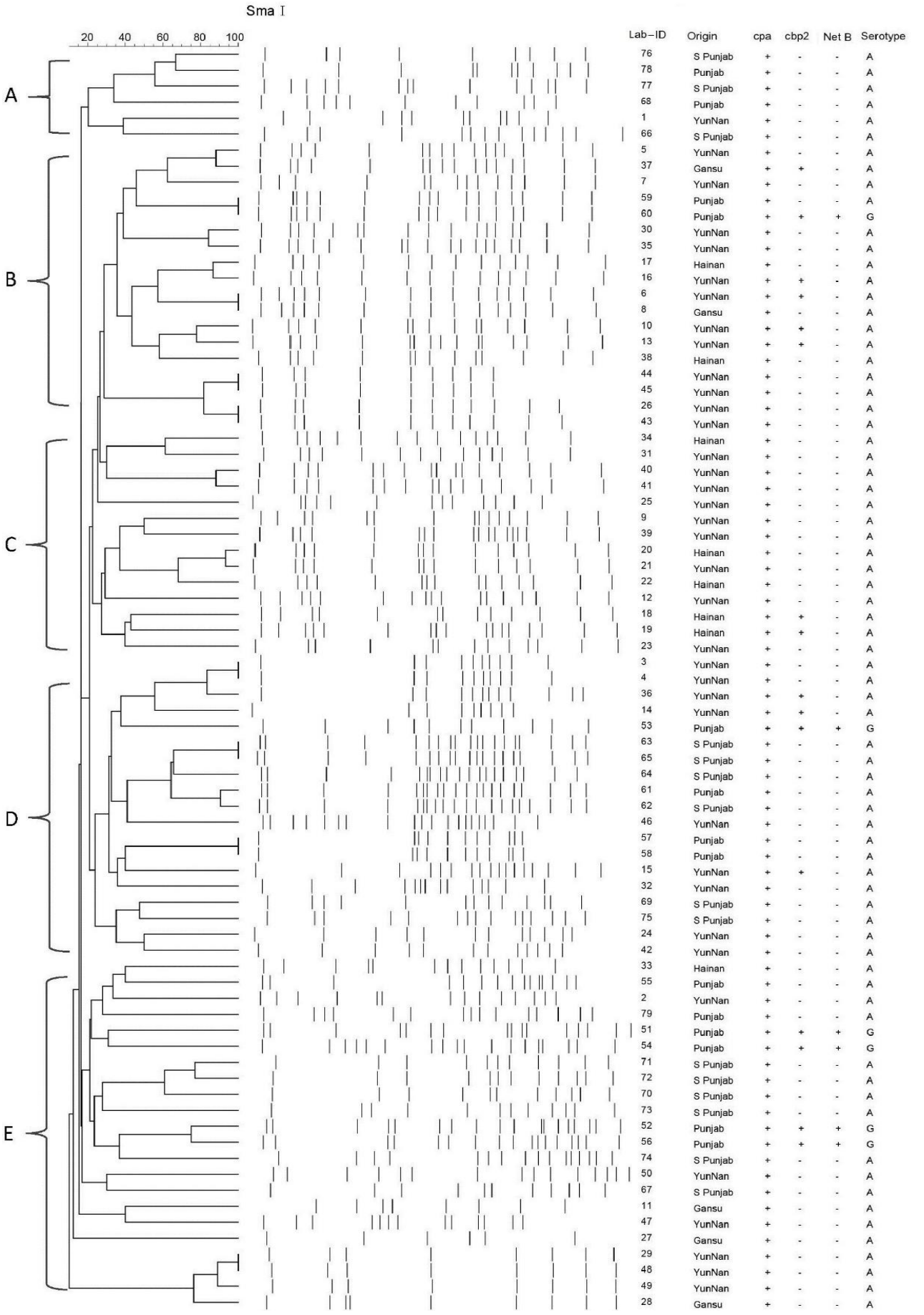

Fig. 4: Schematic representation (genetic profile, isolate identification, toxinotypes, serotype,) of 79 *Clostridium perfringens* isolates obtained from the poultry birds of China and Pakistan. Macro-restriction pattern was conducted with *SmaI*. Dendrogram obtained using BioNumerics 7.6 software and 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 pulsotypes. 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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