Short Communication

Pulsed-field gel electrophoresis of enterotoxic *Clostridium perfringens* type A isolates recovered from humans and animals in Kolkata, India

Jay P. Yadav [a], Suresh C. Das [b],*, Pankaj Dhaka [a], Asish K. Mukhopadhyay [c], Goutam Chowdhury [c], Syamal Naskar [b], Satyaveer S. Malik [a]

[a] *Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar 243 122, India*
[b] *Indian Veterinary Research Institute, Eastern Regional Station, 37, Belgachia Road, Kolkata 700 037, India*
[c] *Division of Bacteriology, National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata 700 010, India*

ABSTRACT

Clostridium perfringens is one of the most important globally recognised gastroenteric pathogen in humans as well as animals. The present study was aimed to know the similarities/divergence among *C. perfringens* type A isolates of human and animal origin using the pulsed-field gel electrophoresis (PFGE) as a molecular tool. The enterotoxic isolates obtained by screening of human diarrhoeal cases (n = 130), diarrhoeal cases of pig (n = 52) and goat (n = 50), meat samples *viz.*, pork (n = 59) and chevon (n = 57) were characterized by standard cultural and biochemical methods followed by PCR Assays. Accordingly, a total of 11 *C. perfringens* type A characterized isolates (16S rRNA$^+$, *cpa*$^+$, *cpb$_2$*$^+$ and *cpe*$^+$) recovered from human diarrhoeal cases (n = 3); diarrhoeal cases of pig (n = 2) and goat (n = 2); meat samples *viz.* pork (n = 2) and chevon (n = 2) were examined employing PFGE. The observed clustering pattern in PFGE analysis showed the relatedness between isolates from diarrhoeal goat and chevon (90–100%); diarrhoeal pig and pork (65–68%); moreover, isolates from human diarrhoeal cases were exhibiting lineage to cases from goat and pig diarrhoea as well pork and chevon by 62–68% relatedness. The outcome of the present study indicates the probable contamination of this pathogen to the human food chain through faeces from suspected food animals *viz.* goat and pig and their improperly cooked meat.

© 2017 Faculty of Veterinary Medicine, Cairo University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Clostridium perfringens is one of the most important pathogen responsible for intestinal infections as well as the histotoxic diseases in humans as well as animals [1]. In developed countries, *C. perfringens* type A food poisoning is one of the most commonly reported food-borne illness, whereas, the disease is not much explored in developing countries, including India [2,3].

Based on the production of four major lethal toxins i.e. alpha (α), beta (β), epsilon (ε) and iota (ι), *C. perfringens* is divided into five major toxinotypes/biotypes (A–E) [4,5]. Apart from these toxins, the pathogen also produces enterotoxin (CPE) and beta2 (β2) toxins (CPB2), which are strongly associated with food poisoning in humans and gastroenteritis in animals [6]. Enterotoxigenic *C.*

perfringens type A is also associated with cases of antibiotic associated diarrhoea (AAD) and sporadic diarrhoea [7,8]. Foodborne isolates of *C. perfringens* harbouring most frequently a chromosomal enterotoxin gene (*cpe*) whereas isolates from patients with AAD, sporadic diarrhoea, as well as in animal diarrhoeal cases usually have a plasmid borne *cpe* gene [4]. The food poisoning associated with *C. perfringens* type A mainly occurs due to the improperly cooked and/or mishandled meat and meat products and may involve large numbers of victims [9]. The source of these food poisoning outbreaks can be traced out employing appropriate epidemiological methods [10]. Among various available molecular typing tools, pulsed-field gel electrophoresis (PFGE) remains one of the most important third-generation molecular typing approach for the detection of genotypic diversity of almost all bacterial species and continues to be recognized as the gold standard due to outcome spanning exceeding 90% of the bacterial genome and its standardized protocols and reagents applicable to a wide range of organisms [11–13]. Globally, limited studies had been carried out to assess the genetic relatedness of *C. perfringens* isolates from different sources [14–21]. But to the best of our knowledge there

are no available studies regarding molecular typing of *C. perfringens* type A isolates through PFGE from different sources from India. With these views, the present investigation was envisaged to assess the genetic relatedness of the *C. perfringens* type A isolates recovered from samples of human and animal origin using PFGE as a molecular tool and thereby, to ascertain the possibility of their intersource transmission/contamination.

2. Materials and methods

2.1. Sample collection and area of the study

In the present study stool/faecal samples from human diarrhoeal cases (n = 130), diarrhoeal pig (n = 52), diarrhoeal goat (n = 50), and meat samples *viz.* pork (n = 59) and chevon (n = 57) were collected to know the prevalence of enterotoxigenic *C. perfringens* type A. The human diarrhoeal samples were collected from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India in screw cap polypropylene tubes containing Cary-Blair transport medium by following the ethical guidelines. Animal samples were collected from organised and unorganised pig and goat farms and butcher's herds in and around the area of humans sample source using sterile screw cap glass tubes. The pork and chevon samples were collected in sterile plastic sachet. Animal samples were also collected with the consent of the concerned authorities, whenever required. The collected samples were immediately transported to the laboratory using ice pack container and processed within 24 h for isolation and identification of *C. perfringens* type A using standard bacteriological and molecular procedures *viz.*, PCR Assays.

2.2. Enterotoxigenic C. perfringens type A isolates

A total of 11 enterotoxigenic *C. perfringens* type A isolates (16S rRNA[+], *cpa*[+], *cpb2*[+] and *cpe*[+]) obtained from human diarrhoeal cases (n = 3), diarrhoeal cases of pig (n = 2) and goat (n = 2) and their meat *viz.*, pork (n = 2) and chevon (n = 2), that were closely linked to the vicinity of human diarrhoeal patients, were subjected to PFGE in order to assess the relatedness between the isolates and the possibility of cross contamination. The PFGE was performed following the PulseNet protocol [22]. In brief, test strains grown on sulphite polymyxin sulphadiazine (SPS) agar (HiMedia, India) were inoculated in Robertson cooked meat medium (RCM) broth (HiMedia, India) and incubated overnight at 37°C under anaerobic condition. Then the overnight culture was suspended in Cell Suspension Buffer (CSB) (100 mM Tris:100 mM EDTA, pH 8.0) and measured the cell concentration in UV-Spectrophotometer by taking the OD value between 1.3 and 1.5 at 600 nm.

2.3. PFGE analysis

The agarose plugs were prepared by mixing equal volume of bacterial culture solution with 1% low-melting agarose (SeaKem). After solidification, the plugs were dressed following instructions with proper cutter. The bacterial cells in the agarose plugs were lysed by treatment with cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) and treated with proteinase K (20 mg/mL) at 54°C for 1 h with constant and vigorous agitation (150–175 rpm).

The plugs were washed twice with sterile ultrapure water (pre-heated to 50°C) under vigorous shaking in a 50°C water bath for 10–15 min each time and further washed 4 times similarly with pre-heated (50°C) TE buffer (10 mM Tris:1 mM EDTA, pH 8.0). Agarose plugs containing genomic DNA were equilibrated in TE buffer and were placed in 30 μL of 10× H buffer (0.1% BSA, 0.1% Triton X-100) [pre-incubation for digestion] for 45 min. After incuba-

tion each plug was kept overnight in 150 μL reaction mixture consisting 15 μL 10× H buffer, 15 μL 10× BSA, 3 μL *Sma*I enzyme (45 units) [Takara, Shuzo Co. Ltd, Japan] and 117 μL sterile triple distilled water at 37 °C.

PFGE of the *Sma*I digested inserts was performed by the contour clamped homogeneous electric field method on a CHEF Mapper system (Bio-Rad, California, USA) with 1% PFGE grade agarose in 0.5× TBE (40 mM Tris-HCl, pH 8.3, 45 mM boric acid, 1 mM EDTA) for 24 h using the *Xba*I digested DNA of *Salmonella enteric* serovar Braenderup (H9812) as the standard size DNA molecular marker. A mini chiller (Bio-Rad) was used to maintain the temperature of the buffer at 14°C. Run conditions (150 mA current, voltage-6.0 V/cm, angle-120°, initial switch time-10 s, final switch times-35 s, linear) were generated by the auto-algorithm mode of the CHEF Mapper PFGE system by using a 78–390 kb size range. After electrophoresis, the gel was stained in ethidium bromide (1 μg/mL) for 30 min and destained in water for 15 min twice. The DNA bands were visualized and photographed with the BioSpectrum AC Imaging System (USA). The PFGE analysis has been carried out by using BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between isolates were evaluated by using the cluster analysis with the unweighted pair group method with arithmetic mean (UPGMA) method and the Dice correlation coefficient with a position tolerance of 1.5%.

3. Results

On PFGE analysis, the 11 tested isolates of *C. perfringens* type A (human, pig and goat diarrhoeal cases; pork and chevon) have been clustered into 4 major groups ('A' through 'D') (Fig. 1). The isolates from chevon (CHV2), goat faeces (GF2) and human diarrhoeal cases (IDH 08342) were belonged to the cluster 'A'. Among them, the isolate from chevon (CHV2) and goat faeces (GF2) were grouped in a single clad and showed 100% similarity in band pattern. Further, the human isolate (IDH 08342) was linked to them with a similarity of about 62%. Isolates from four sources i.e., chevon (CHV1), pork (PRK2), goat faeces (GF1) and human diarrhoeal cases (BCH 05751) were grouped in a single cluster (cluster 'B'); where the isolate from chevon (CHV1) and pork (PRK2) belonged to the same clad with 100% similarity; however, the isolate from goat faeces (GF1) was showing the similarity of 90% with chevon (CHV1) and pork (PRK2) isolates. Further, human isolate (BCH 05751) was found to be similar (about 68%) with these three isolates (CHV1, PRK2 and GF1, Fig. 1). Further, the isolates belonging to the cluster 'C' [human diarrhoeal cases (IDH 08341) and pig faeces (PF2)] were
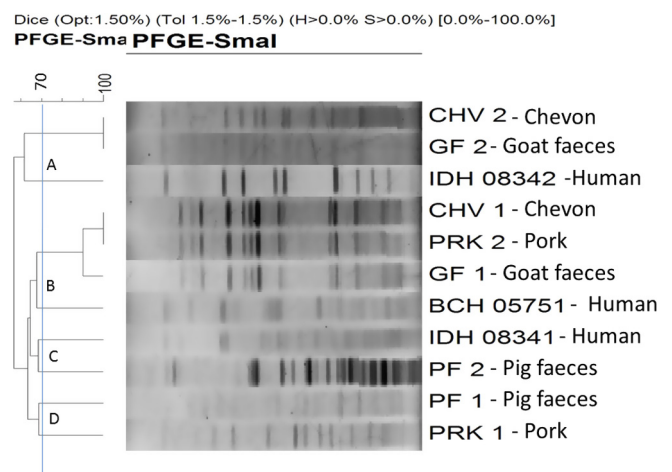

Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-SmaI

A	CHV 2 - Chevon
	GF 2 - Goat faeces
	IDH 08342 - Human
B	CHV 1 - Chevon
	PRK 2 - Pork
	GF 1 - Goat faeces
	BCH 05751 - Human
C	IDH 08341 - Human
	PF 2 - Pig faeces
D	PF 1 - Pig faeces
	PRK 1 - Pork

Fig. 1. PFGE analysis of representative isolates of *C. perfringens* type A.

found to exhibit about 68% similarity. Similarly in the cluster 'D', isolate from pig faeces (PF1) and pork (PRK1) was grouped with about 68% similarity. The isolates belonged to cluster 'B' (chevon, pork, goat faeces and human diarrhoeal cases) were linked to the cluster 'C' (human diarrhoeal cases and pig faeces) with a similarity range of about 65%. Similarly, the isolates from cluster 'D' (pig faeces and pork) were linked to isolates of cluster 'B' (chevon, pork, goat faeces and human diarrhoeal cases) with a similarity range of about 65%. Likewise, the isolates from human and animal origin (cluster 'B', 'C' and 'D') were linked to the cluster 'A' and expressed about 55% similarity (Fig. 1).

4. Discussion

On analysis of dendogram, it was observed that the isolates from chevon (CHV2), goat faeces (GF2) and human diarrhoeal cases (IDH 08342) were grouped into the cluster 'A'. The isolates from chevon (CHV2) and goat faeces (GF2) were grouped in a single clad and showed 100% similarity in band pattern. Besides, the human isolate (IDH 08342) was linked to them with a similarity of about 62%. The belonging of the isolates from goat faeces and chevon in the same clad and band pattern similarity strongly suggests the possibility of contamination of this pathogen from food animal (goat) to its meat (chevon). This might be possible for the unhygienic dressing of carcass and its transportation. Further, the human diarrhoeal isolate (IDH 08342) showed the similarity of about 62% to the isolate from the goat faeces (GF2) and chevon (CHV2) which support the probable entry of the pathogen to human food chain through suspected goat meat (chevon). The findings of the present study were concurrent with earlier study reports [18,21,23].

In cluster 'B' isolates from four sources i.e., chevon (CHV1), pork (PRK2), goat faeces (GF1) and human diarrhoeal cases (BCH 05751) were grouped and the isolates from chevon (CHV1) and pork (PRK2) were belonged to the same clad with 100% similarity; however, the isolate from goat faeces (GF1) showed similarity of 90% with chevon (CHV1) and pork (PRK2) isolates. Moreover, one human isolate (BCH 05751) was expressed about 68% similarity with these three isolates (CHV1, PRK2 and GF1). The findings showed the significant possibility for passing of this pathogen from goat to its meat. Moreover, belonging of the isolates from two meat sources i.e., chevon and pork in a same clad with 100% similarity draws attention for the possibility of cross-contamination, even these two different types of meat are separately prepared from two different type of food animals i.e., goat and pig. This could be attributed to unhygienic handling of meat during marketing chain. The genetic diversity of C. perfringens type A isolates from different sources were found to be in concurrent with the observation of genetic variation among C. perfringens isolates by using PFGE tool by others researchers [21,24].

Isolates from human diarrhoeal cases (IDH 08341) and pig faeces (PF2) were found to exhibit about 68% similarity in cluster 'C'. Similar to the cluster 'A' and 'B' observations, the findings in cluster 'C' also expressed the strong possibility of transmission of this pathogen from pig to human or vice versa. Each isolate from pig faeces (PF1) and pork (PRK1) was grouped in cluster 'D' and showed about 68% similarity among them. As revealed in cluster 'A', the findings suggesting the probable route of contamination of this pathogen from food animal (pig) to its meat (pork). The findings of present study were concurrent with earlier study reports [18,20].

The analysis of PFGE findings of the isolates from human and animal sources clearly revealed their relatedness in a range of 62–68%. This indicates the probable circulation of this pathogen to the human food chain through faeces of suspected food animals viz. goat and pig and their improperly cooked meat. The present study was in accord with the earlier studies using PFGE as an epidemiological tool to track the source of infection in case of C. perfringens [16,19–21,25,26].

5. Conclusions

The present study involving PFGE analysis of the tested C. perfringens type A isolates revealed the probability of contamination of meat with unhygienic slaughter and meat processing practices. It is well established that this pathogen and its enterotoxin are labile to the degree of temperature attained in the Indian traditional cuisine. The essence of the present study suggests that the adoption of standard cooking methods is indispensable to evade health hazards with this food borne zoonotic bacterial pathogen.

Acknowledgement

Competing interests

The authors declare that there are no competing interests.

References

[1] Ezzeldeen NA, Ammar AM, Shalaby B, Haririr ME, Omar WS. Rapid detection of Clostridium perfringens in seafood. Adv Environ Biol 2016;10:174–81.
[2] Lindström M, Heikinheimo A, Lahti P, Korkeala H. Novel insights into the epidemiology of Clostridium perfringens type A food poisoning. Food Microbiol 2011;28:192–8.
[3] Yadav JP, Das SC, Dhaka P, Vijay D, Kumar M, Mukhopadhyay AK, et al. Molecular characterization and antimicrobial resistance profile of Clostridium perfringens type A isolates from humans, animals, fish and their environment. Anaerobe 2017;47:120–4.
[4] Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, et al. Toxin plasmids of Clostridium perfringens. Microbiol Mol Biol Rev 2013;772:208–33.
[5] Silva ROS, Lobato FCF. Clostridium perfringens: a review of enteric diseases in dogs, cats and wild animals. Anaerobe 2015;33:14–7.
[6] Fisher DJ, Miyamoto K, Harrison B, Akimoto S, Sarker MR, McClane BA. Association of beta2 toxin production with Clostridium perfringens type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol Microbiol 2005;56:747–62.
[7] Banaszkiewicz A, Kądzielska J, Gawrońska A, Pituch H, Obuch-Woszczatyński P, Albrecht P, et al. Enterotoxigenic Clostridium perfringens infection and pediatric patients with inflammatory bowel disease. J. Crohns Colitis 2014;8:276–81.
[8] Freedman JC, Shrestha A, McClane BA. Clostridium perfringens enterotoxin: action, genetics, and translational applications. Toxins 2016;8:73.
[9] Lund BM, Baird-Parker TC, Gould GW. The microbiological safety and quality of food. Gaithersburg, MD, USA: Aspen Publishers; 2000.
[10] Palmer SR. Epidemiological methods in the investigation of food poisoning outbreaks. Lett Appl Microbiol 1990;11:109–15.
[11] Nassonova ES. Pulsed field gel electrophoresis: theory, instruments and application. Cell Tissue Biol 2008;2:557–65.
[12] Goering RV. Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. Infect Genet Evol 2010;10:866–75.
[13] Goering RV, Fey PD. Pulsed field gel electrophoresis of Staphylococcus epidermidis. Methods Mol Biol 2014;11:55–60.
[14] Maslanka SE, Kerr JG, Williams G, Barbaree JM, Carson LA, Miller JM, et al. Molecular subtyping of Clostridium perfringens by pulsed-field gel electrophoresis to facilitate food-borne-disease outbreak investigations. J Clin Microbiol 1999;37:2209–14.
[15] Schalch B, Sperner B, Eisgruber H, Stolle A. Molecular methods for the analysis of Clostridium perfringens relevant to food hygiene. FEMS Immunol Med Microbiol 1999;24:281–6.
[16] Lukinmaa S, Takkunen E, Siitonen A. Molecular epidemiology of Clostridium perfringens related to food-borne outbreaks of disease in Finland from 1984 to 1999. Appl Environ Microbiol 2002;68:3744–9.
[17] Schalch B, Bader L, Schau HP, Bergmann R, Rometsch A, Maydl G, et al. Molecular typing of Clostridium perfringens from a food-borne disease outbreak in a nursing home: ribotyping versus pulsed-field gel electrophoresis. J Clin Microbiol 2003;41:892–5.

[18] Johansson A, Aspan A, Bagge E, Båverud V, Engström BE, Johansson KE. Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. BMC Microbiol 2006;6:47.

[19] Ferreira TSP, Moreno AM, Almeida RRD, Gomes CR, Gobbi DDSD, Filsner PHNDL, et al. Molecular typing of *Clostridium perfringens* isolated from swine in slaughterhouses from São Paulo State, Brazil. Cienc Rural 2012;42:1450–6.

[20] Lee KE, Lim SI, Shin SH, Kwon YK, Kim HY, Song JY, et al. Distribution of *Clostridium perfringens* isolates from piglets in South Korea. J Vet Med Sci 2014;76:745.

[21] Park M, Deck J, Foley SL, Nayak R, Songer JG, Seibel JR, et al. Diversity of *Clostridium perfringens* isolates from various sources and prevalence of conjugative plasmids. Anaerobe 2016;38:25–35.

[22] CDC. www.cdc.gov/pulsenet/.../PulseNetAsia_Pacific; 2009.

[23] Brzychczy-Włoch M, Bulanda M. Analysis of genetic similarities between *Clostridium perfringens* isolates isolated from patients with gas gangrene and from hospital environment conducted with the use of the PFGE method. Pol J Surg 2014;86:141–6.

[24] Park JY, Kim S, Oh JY, Kim HR, Jang I, Lee HS, et al. Characterization of Clostridium perfringens isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poult Sci 2015;94:1158–64.

[25] Lahti P, Heikinheimo A, Johansson T, Korkeala H. *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (Genotype IS*1151*-*cpe* or IS*1470*-like-*cpe*) as a common cause of food poisoning. J Clin Microbiol 2008;46:371–3.

[26] Johansson A, Aspán A, Kaldhusdal M, Engström BE. Genetic diversity and prevalence of netB in *Clostridium perfringens* isolated from a broiler flock affected by mild necrotic enteritis. Vet Microbiol 2010;144:87–92.