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Short Communication

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

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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.

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

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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 incubation 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

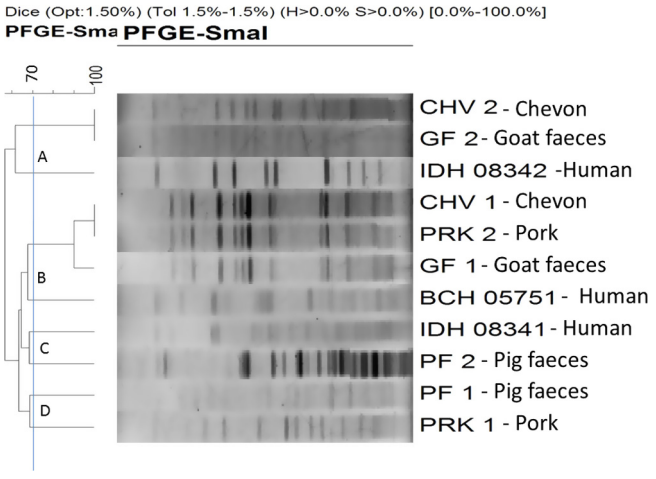

Fig. 1. PFGE analysis of representative isolates of *C. perfringens* type A.

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