Genomic diversity of *Clostridium perfringens* strains isolated from food and human sources

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Summary

Clostridium perfringens is a serious pathogen which causes enteric diseases in domestic animals and food poisoning in humans. Spores can survive cooking processes and play an important role in the possible onset of disease. In this study, RAPD-PCR and REP-PCR were used to examine the genetic diversity of 49 isolates of *C. perfringens* type A from three different sources. The results of RAPD-PCR revealed the most genetic diversity among poultry isolates, while human isolates showed the least genetic diversity. Cluster analysis obtained from RAPD-PCR and based on the genetic distances split the 49 strains into five distinct major clusters (A, B, C, D, and E). Cluster A and C were composed of isolates from poultry meat, cluster B was composed of isolates from human stool, cluster D was composed of isolates from minced meat, poultry meat and human stool and cluster E was composed of isolates from minced meat. Further characterization of these strains by using (GTG) 5 fingerprint repetitive sequence-based PCR analysis did not show further differentiation between various types of strains. In conclusion, RAPD-PCR method seems to be very promising for contamination source tracking in the field of food hygiene.

Key words: *Clostridium perfringens*, Genetic diversity, RAPD-PCR, REP-PCR

Introduction

Clostridium perfringens has been classified into 5 types (A-E) on the basis of its ability to produce ≥1 of the major lethal toxins, alpha (*α*), beta (*β*), epsilon (*ε*) and iota (*i*) (McClane, 2001). Enterotoxin producing *C. perfringens* (*cpe*+) type A is reported continuously as one of the most common food poisoning agents in the world (Adak *et al*., 2002). *Cpe* positive *C. perfringens* can access the food chain with unclear source and route of contamination (Wen and McClane, 2004). Human and food animals are important reservoirs of *cpe*-positive *C. perfringens*, introducing a risk into foods, primarily through contamination or miss-handling of meat (Lindstrom, 2011). Several typing methods have been used for determining the relatedness of various *C. perfringens* isolates from humans, animals, and foods. These typing methods include, serotyping (Gross *et al*., 1989), bacteriocin typing (Schalch *et al*., 1998), phage typing (Yan, 1989), plasmid profile typing (Schalch *et al*., 1998; Schalch *et al*., 1999), ribotyping (Schalch *et al*., 2003), pulse field gel electrophoresis (PFGE) (Nakamura *et al*., 2003; Schalch *et al*., 2003), multilocus sequence typing (MLST) (Jost *et al*., 2006), randomly amplified polymorphic DNA (RAPD) (Leflon *et al*., 1997), repetitive element PCR (rep-PCR) (Siragusa *et al*., 2006), amplified fragment length polymorphism (AFLP) (McLauchlin *et al*., 2002; Settings, 2006). Each method has specific characteristics, for example, they are not always available in the microbiological laboratory, they may lack typeability, they are technically fastidious, or time consuming. Random amplified polymorphic DNA PCR analysis is a rapid technique, easily applicable in clinical laboratories, and a suitable tool for epidemiological studies (Leflon *et al*., 1997). Another simple method is determining oligonucleotide repeat, (GTG) 5, which can be used to rapidly classify or accurately genotype species from various genera, including non tuberculous *Mycobacterium* spp. (Cilliers *et al*., 1997), *Enterococcus* spp. (Svec *et al*., 2005), and *Lactobacillus* spp. isolates (Gevers *et al*., 2001).

In this study, persons with gastroentric disease were screened for faecal carriage of different types of *C. perfringens* and determining the *cpe* positives isolates. The genetic relationship between *C. perfringens* isolates, obtained from different sources (human and meat) was also assessed with two genotyping methods, RAPD-PCR and REP-PCR.

This appears to be the first report to evaluate the comparative efficacy and to assess the origin tracking potential of Rep-PCR and RAPD-PCR methods in molecular discrimination of *C. perfringens* isolates obtained from different sources.

Materials and Methods

Samples

Isolates of *C. perfringens* which were included in this study were isolates from broiler meat (N=22) and minced red meat (N=9) in our previous study (Afshari *et al*.,

2015a, b), and 20 human source isolates which were obtained from Microbiology Laboratory of Ghayem Hospital Clinic, in the northeast of Iran during winter of 2013. The patients suffered from mild to severe gastrointestinal symptoms at the time of stool sampling.

For confirmation, all isolates were streaked onto sheep blood agar and incubated at 37°C for 24 h in an anaerobic jar (Merck, Darmstadt, Germany). Colonies with double zone hemolysis and characteristic morphology on 10% sheep blood agar were selected for further confirmation by 16S rDNA PCR according to Wu *et al*. (2009).

Typing by multiplex PCR

DNA extraction of human *C. perfringens* isolates was performed and subjected to multiplex PCR to determine the toxinotypes (A-E) and also the presence of *cpe* and *cpb2* gene according to (Meer and Songer, 1997) which was described in detail previously(Afshari *et al*., 2015a, b).

RAPD genotyping

DNA amplification was carried out by using 1 µL of the lysate in 500 mM KCl, 10 mM Tris HCl, 2.5 mM $MgCl_2$, containing 400 µM of each dNTP, 0.5 U of *Taq* DNA polymerase (Thermo Scientific Fermentase, USA) and 4 µM of primer, in a final volume of 25 µL. Amplification was performed in a thermocycler (Techne TC, 3000, England) with the amplification conditions of 94°C for 15 s, 36°C for 15 s, 72°C for 70 s for 44 cycles and 72°C for 5 min for the last cycle (Leflon *et al*., 1997). PCR products were analyzed by electrophoresis in 1.4% agarose gels containing ethidium bromide and photographed under UV light. For each primer (HLWL74 and MHN1) (Leflon *et al*., 1997), the amplification was independently performed twice the same day, and once more another day to evaluate the repeatability and the reproducibility of the method, respectively.

(GTG) 5-rep-PCR fingerprinting

A total of 49 type A of *C. perfringens* isolates were subjected to repetitive sequence-based PCR (rep-PCR) fingerprinting with the primer (GTG) 5 (Gevers *et al*., 2001; Xiao *et al*., 2012). PCR amplifications were performed in a total volume of 25 µL: 12.5 µL of the ampliqon 2 × master mix red (Odense M, Denmark) contains: Tris-HCl pH = 8.5, $(NH4)_2S0_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM of each dNTP, 0.2 units/µL Ampliqon Taq DNA polymerase, 100 ng DNA template, 1 µM (GTG) 5 primer. The remainder of the reaction volumes includes water. Amplification reactions were as described previously (Versalovic *et al*., 1994). DNA fragments were separated by gel electrophoresis using a 1% agarose gel.

Statistical analysis

Scanned images were analyzed using Photo Cap. software. Bands were assigned on a presence-absence basis. The software estimated band sizes for RAPD-PCR and REP-PCR data. The data were analyzed using SPSS software, version 16. Because the data were binary, using jaccard distance matrix and Ward's hierarchical cluster technique, isolates were clustered and displayed in dendrogram form.

Results

Typing of toxigenic *C. perfringens* strains, isolated from human stool, using multiplex PCR assay revealed that type (A) was the most predominant type (90%) compared to the other type of *C. perfringens*, with the incidences of 10% for type D, respectively. Our results showed that, out of 18 type A isolates, 2 isolates carried the *cpb2* gene (11%). Toxin typing of broiler meat and minced meat isolates was performed previously (Afshari *et al*., 2015a, b).

RAPD-PCR

Amount of 49 *C. perfringens* isolates recovered from human stool (n=18), broiler meat (n=9) and minced meat (n=22), were typed using short sequence primers. Forty six isolates out of 49 isolates were typeable by this typing method. The RAPD-PCR profile analysis of human stool isolates showed that HLWL74 primer identified 8 bands that varied from 150-1200 bp and MHN1 primer identified 7 bands that varied from 100-500 bp. The RAPD-PCR profile analysis of broiler meat isolates showed that the HLWL74 primer identified 7 bands that varied from 160-700 bp and MHN1 primer identified 6 bands that varied from 130-450 bp. The RAPD-PCR profile analysis of minced meat isolates showed that HLWL74 primer identified 4 bands that varied from 250-700 bp and MHN1 primer identified 13 bands that varied from 100-600 bp.

Both primers produced a total of 18 bands across 22 isolates of minced meat, 14 bands across the 18 isolates of human stool and 10 bands across the 9 isolates of poultry.

Of these bands, 6 bands were polymorphic, 4 bands were common (monomorphic band) and no band was specific in poultry isolates. In minced meat isolates, 2 bands were polymorphic, 14 bands were common and 1 band was specific. In human isolates, no polymorphic band, 9 common bands and 3 specific bands were observed.

Cluster analysis based on the genetic distances split the 49 strains into five distinct major clusters (A, B, C, D, and E) (Fig. 1) with obvious association between the pattern of clustering of the strains and their host specificities. The first and the third clusters (A and C) included 100% of poultry strains, second cluster (B) included 100% of human strains, cluster D included 57.1% (12 isolates) of human strains, 38% (8 isolates) of minced meat strains and 4.7% (1 isolates) of poultry strains and cluster E included 100% of minced meat strains.

(GTG) 5-rep-PCR fingerprinting

By using (GTG) 5-rep-PCR fingerprinting, 39

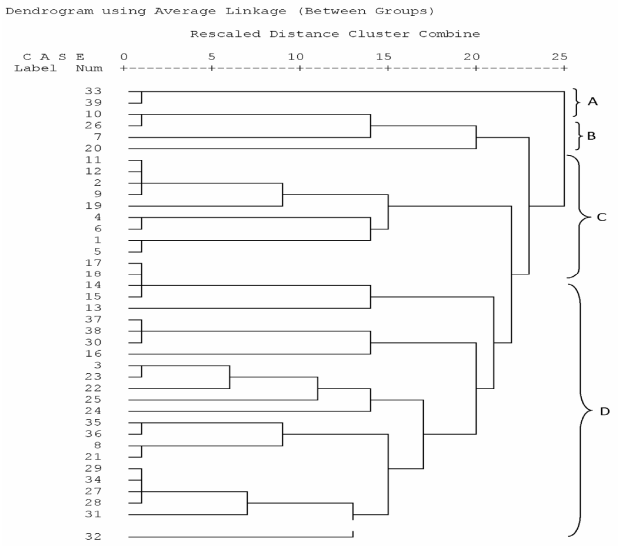

Fig. 1: Dendrogram using average linkage (between groups) of toxigenic *C. perfringens* isolates from 3 different sources (1-18 human stool isolates, 19-24 broiler meat isolates, and 25-46 sheep+calf minced meat isolates) by using RAPD-PCR method

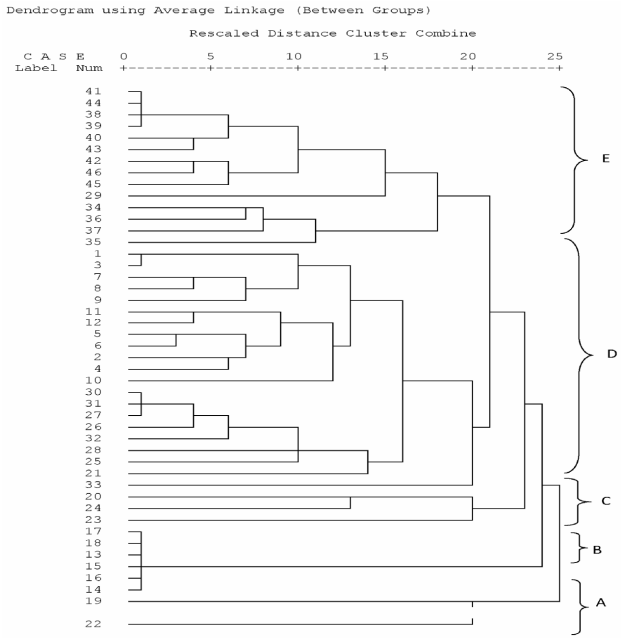

Fig. 2: Dendrogram using average linkage (between groups) of toxigenic *C. perfringens* isolates from 3 different sources (1-18 human stool isolates, 19-26 broiler meat isolates, and 27-39 sheep+calf minced meat isolates) by using REP-PCR method

isolates out of 49 isolates were typeable. The REP-PCR profile analysis of human stool isolates showed that (GTG) 5 primer identified 6 bands varying from 200-900 bp, in broiler meat isolates, 6 bands that varied from 200-1200 bp and in minced meat isolates, 4 bands varied from 300-900 bp were identified. Based on (GTG) 5-rep-PCR fingerprinting patterns of all 49 identified *C. perfringens* type A strains, cluster analysis showed four distinct major clusters (A, B, C, and D). Cluster A included 100% of minced meat strains, cluster B included 50% (2 isolates) of poultry strains and 50% (2 isolates) of human strains, cluster C included 88% (8 isolates) of human strains and 11% (1 isolate) of poultry strains and cluster D included 41.6% (10 isolates) of minced meat strains, 25% (6 isolates) of poultry strains and 33.3% (8 isolates) of human strains (Fig. 2).

Discussion

Understanding the reservoirs and routes of transmission of *C. perfringens* type A strains causing food poisoning is very important in epidemiological investigations, as it was found to be the third most common cause of US bacterial food-borne disease, following *Campylobacter* and *Salmonella* spp., (Novak and Juneja, 2002; Moffatt *et al*., 2011; Wahl *et al*., 2013). The relative levels of *C. perfringens* type A in different sources like human stool and animal production, especially meat, is thought to be helpful in establishing a causal role for this bacterium in cases of food poisoning.

In this study none of the type A isolates from different sources carry the *cpe* gene, despite the emphasis that human stool is one of the most important sources of *cpe* positive *C. perfringens*. Heikinheimo *et al*. (2004) reported the prevalence of 18% for enterotoxin gene-carrying (*cpe*+) of *C. perfringens* in the faeces of healthy food handlers. Because a small number of entero-toxigenic *C. perfringens* with a large number of non enterotoxigenic ones co-exist in faeces, if a limited number of isolates from faecal samples are cultured for screening enterotoxigenicity, enterotoxigenic strains may not be detected (Miwa *et al*., 1997). The other possibility that *cpe* gene was not detected, might be the immuno-compromised nature or age of the examined persons in this study who were mostly middle-aged persons. The presence of *C. perfringens* type A even without harboring *cpe* gene can also be notable because *cpe* gene is mobile and can transfer from plasmid to chromosome and thus result in food born disease (Ma *et al*., 2012).

However, adoption of proper prevention and control measures including cooking procedures, hygiene, strict adherence to HACCP principles, public awareness and disease surveillance and monitoring are important.

In this study toxin typing of *C. perfringens* isolates from human stool showed 90% type A and 10% type D. Carman *et al*. (2008) detected only type A of *C. perfringens* in normal faecal flora of healthy North Americans. Another gene which was investigated in our study was *cpb2* gene, which is a necrotizing and lethal toxin and can be produced by all types of *C. perfringens*, and has been blamed for disease in many animal species and human (Uzal *et al*., 2010). *Clostridium perfringens* isolates carrying *cpb2* are recognized in human gastrointestinal diseases, including food poisoning, antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD) (Van Asten *et al*., 2010). In a study on faecal samples of 43 healthy North Americans, *C. perfringens* harboring *cpb2* gene was isolated from 13 samples (Carman, 2008). In our study some of the type A isolates from human (11.1%) carried the *cpb2* gene.

The evaluation of the relationship between sources of *C. perfringens*, especially for the purpose of source tracking, is of great importance. RAPD-PCR method facilitates study of changes in diversity over time and can be a basis for subsampling in large populations. Many studies have shown that this method is a rapid and reproducible technique and can distinguish closely related species of bacteria (Williams *et al*., 1990; Barbut *et al*., 1993; Power, 1996; Leflon *et al*., 1997). In the present work, an attempt was made to use the RAPD technique to investigate the relatedness among 49 type A isolates of *C. perfringens* and to generate a molecular fingerprint of the bacterium from three different sources (broiler meat (n=9), sheep+calf minced meat (n=22) and human stool (n=18)), using 2 short sequence primers. All Strains were divided into 5 major groups based on genomic diversity. Leflon-Guibout *et al*. (1997) developed a RAPD system in comparison with zymotyping. Thirteen primers were tested and it was concluded that the RAPD typing which is less fastidious than zymotyping can be used as an epidemiological marker for *C. perfringens*. Baker *et al*. (2006) used RAPD-PCR method to evaluate the diversity of toxigenic *C. perfringens* and *Clostridium difficile* among swine herds in the Midwest. As expected, both species appeared to be genetically diverse.

Further characterization of 49 type A of *C. perfringens* by using (GTG) 5 fingerprint repetitive sequence-based PCR analysis divided broiler meat, minced meat and human stool isolates into 4 major groups A, B, C, and D. In a similar study, Xiao *et al*. (2012) distinguished *C. perfringens* from other sulfite-reducing Clostridia by REP-PCR method but this method was not able to differentiate various types of *C. perfringens* strains. Northey *et al*. (2005) showed an excellent correlation between REP-PCR subtypes and PFGE subtypes and both methods displayed broadly similar discriminatory powers.

Both of the typing methods employed were able to distinguish among isolates from particular sources. However, on the basis of a comparison of the results from these assays, RAPD-PCR analysis was more discriminatory than REP-PCR for the isolates studied. Cross contamination was assessed based on the similarities of the fingerprints of *C. perfringens* isolated from the different sources. Data obtaining may be useful in assessing the health risk of these contaminated food products to consumers in this area. Based on the results from this study, isolates from different sources may have originated from a single clone and transmitted through the meat consumption from animal to human.

Different typing methods can discriminate and reveal close similarities between isolates from different sources in properly managed and poorly designed public health systems. It is therefore suggested protective measures during food processing to reduce the risk of human infection.

In conclusion RAPD-PCR method seems to be very promising for the epidemiological investigation of food-borne diseases caused by *C. perfringens* and for contamination source tracking in the field of food hygiene and industry.

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Conflict of interest

The authors declare no conflict of interest.

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