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Molecular characterization of *Clostridium perfringens* isolated from cattle and sheep carcasses and its antibiotic resistance patterns in Shiraz slaughterhouse, southern Iran

Saeid Hosseinzadeh[1]*, Maryam Bahadori[2], Maryam Poormontaseri[1], Maryam Dehghani[3], Mahdi Fazeli[3], and Saeed Nazifi[4]

[1]*Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

[2]*Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

[3]*Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

[4]*Department of Clinical Studies, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

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ABSTRACT

Clostridium perfringens type A food-borne poisoning is often caused by *C. perfringens* enterotoxin (CPE) encoded by chromosomal *cpe*. Contamination of meat with *C. perfringens* usually leads to food poisoning outbreaks. To find more information regarding the causative agent, we focused on the identification of type A containing *cpe* and *netB* genes in cattle and sheep carcasses slaughtered at Shiraz slaughterhouse and investigated the prevalence of antibiotic-resistant plasmid in isolated *C. perfringens*. 200 specimens were randomly collected by swabbing the whole outer and inner surface of the carcasses, and processed for selective culture on sulfadiazine polymyxin sulphate agar (SPS). The suspected colonies were further identified using species-specific primers as to confirm the presence of the *cpa*, *cpe*, *netB* and tetracycline and enrofloxacin gene resistance patterns. Our results demonstrated that out of 90 and 70 colonies of the positive cultures from cattle and sheep samples, respectively, 40% and 35.7% of the suspected colonies were identified as *C. perfringens* type A by PCR assay. Moreover, from those type A isolates, only 1 (2.7%) isolate was positive for both *cpe* and *netB* genes in the cattle carcasses. The MIC values also showed high tetracycline resistance patterns for cattle (45.8%) and sheep (92.3%) while all of the PCR positive *C. perfringens type A* isolates were susceptible to enrofloxacin. The high prevalence of *C. perfringens* in slaughtered animals with a high rate of resistance to tetracycline implies the need for caution in the use of antibiotic in food animals.

Key words: *Clostridium perfringens*; *cpe* and *netB* genomes; antibiotic resistance

*Corresponding author:

S. Hosseinzadeh, PhD, DVM, Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran, P.O. Box: 71441-69155, Iran, Phone: +98 713 6138 743; Fax: +98 713 228 6950; E-mail: hosseinzadeh@shirazu.ac.ir

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Introduction

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacterium, and an important cause of food-borne gastrointestinal (GI) diseases in both humans and animals (SONGER 1996; WEN and McCLANE 2004). The bacteria persist in nature and can be found in soil, and the intestinal tracts of human, animals and insects. Owing to being a spore-forming bacterium, *C. perfringens* is very resistant to external agents, which in turn favors their distribution (LINDSTROM et al., 2011). Different kinds of *C. perfringens* toxins, including enterotoxin (CPE), have been identified by serological and molecular methods (EL-MOEZ et al., 2013). *C. perfringens* type A, carrying chromosomal *cpe*, is the major cause of food poisoning in the world; while the majority of non-food borne gastrointestinal diseases such as sporadic diarrhea and antibiotic-associated diarrhea are identified as plasmid-borne *cpe*. The reservoirs and transmission routes of enterotoxigenic *C. perfringens* are not entirely clear. Raw meat and poultry are typically considered as the major vehicles for the transmission of this enteropathogen (LAHTI et al., 2008; LINDSTROM et al., 2011). Moreover, *C. perfringens* may cause necrotic enteritis as an economic serious disease in chickens, followed by a pore forming toxin (NetB). A high prevalence of the toxin gene *net*B has been reported in slaughtered animals (MARTIN and SMYTH 2009).

Considering the ubiquitous nature and high load of *C. perfringens* in the GI tract of humans and animals, and also its resistance to multiple antimicrobial agents, it may play a role in the transfer of resistant plasmids to other gut microflora. On the other hand, the similarity of the genotypic and phenotypic characteristics of foodborne bacteria to the resistant bacteria of the GI tract is regarded as a cause of conjugative transfer of the resistant mobile genetic elements to each other. Moreover, the high amount of foodborne pathogens in animal products and the irregular use of anti-bacterial agents may lead to an increase in the rate of antibiotic resistance worldwide (TEUBER, 1999). However, previous publications have shown an increasing pattern of resistance, especially in meat products in developing countries (VAN et al., 2007). In cases of enterotoxigenic *C. perfringens* type A food poisoning, although antibiotic therapy is not of significant value, the presence of high frequencies of antibiotic resistant isolates may adversely affect the gut microflora (TEUBER, 1999). In the current study, we tried to determine the genotypic and phenotypic prevalence of *C. perfringens* carrying *cpe* and *net*B genes, and also identify multi-drug resistance patterns in the slaughtered animals.

Materials and methods

Sample collection and culture. From October 2012 to June 2013, 200 swab samples were randomly collected in thioglycolate broth from cattle (n = 100) and sheep (n = 100) carcasses at a Shiraz slaughterhouse, in the south of Iran. Sampling was carried out by

swabbing the whole outer and inner surfaces of the carcasses (SMART et al., 1979). The samples were immediately transferred to the laboratory on ice and sub-cultured anaerobically on selective culture media, sulfite polymyxin sulfadiazine (SPS) agar, followed by incubation at 37 °C for 24 h. Black colonies were considered as suspicious positive and were then identified by Gram's stain. The suspected colonies were chosen for DNA extraction and PCR assay using species-specific primers targeting the *cpa, cpe,* and *netB* genes in order to perform toxin typing (WU et al., 2009).

Animal ethics. The experiment was performed under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). The European Council Directive (86/609/EC) of November 24, 1986, ethic approval was employed in all the experiments.

DNA extraction. This was performed using the phenol-chloroform technique (SAMBROOK and RUSSELL 2006). The samples were centrifuged at 10000 *g*; the supernatants were discarded; and 250 µL buffer 1 (resuspension solution contained 100 µg/mL RNase), 250 µL buffer 2 (Lysis buffer), and 550 µL saturated phenol were then added, followed by mixing and centrifuging at 8000 *g*. The supernatant of each sample was collected into a new micro tube; the same volume of phenol was then added and centrifuged at the same speed. The clear phase was collected into a new tube, before adding sodium acetate (2M, pH 5.2, 0.1X volume of each aliquot). The aliquot was mixed with 1.5 mL of 100% ethanol, kept at -20 ºC for 1 hour, and centrifuged at 12000 g. The supernatant was then discarded and the DNA pellet was washed with counterpart 80% ethanol, before being dried and suspended in 30 µL distilled water for further use.

Table 1. Primers used in the present study

Primer name	Nucleotides (5'-3')	Annealing temperature (ºC)	Amplicon size (bp)	References
cpa F *cpa* R	GCTAATGTTACTGCCGTTGACC TCTGATACATCGTGTAAG	55	324	WU et al., 2009
cpe F *cpe* R	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	55	233	MIKI et al., 2008
*net*B F *net*B R	GCTGGTGCTGGAATAAATGC TCGCCATTGAGTAGTTTCCC	55	383	TOLOOE et al., 2011
*tet*A F *tet*A R	GAAGGCAAGCAGGATGTAG GAAGGCAAGCAGGATGTAG	50	888	SCHWAIGER et al., 2010
*gyr*A F *gyr*A R	ATGAGCGAATTAGCCAAAGA GCAACCGTCCAACACTTCAT	52	582	WANG et al., 2010

PCR assay. Amplification of cpa gene. The presence of *C. perfringens* type A was confirmed by determination of the *cpa* gene (Table 1). Details of the primers and PCR cycle

are given as follows: the PCR mixture was prepared in total volume of 25 µL containing MgCl2 (2 mM), 10x PCR buffer (2.5 µL), dNTPs (200 mM), forward and reverse primer (500 µM), and Taq DNA polymerase (2 units) (CinnaGen, Iran). PCR amplification was carried out as reported formerly (WU et al., 2009). After electrophoresing the amplified products in 1.5% agarose gel (Cinna Gen, Iran) and staining them with ethidium bromide, and a UV light was used for photographing.

Amplification of cpe gene. The detection of *C. perfringens* containing a *cpe* amplicon was based on the amplification of the gene using oligonucleotide primers (Table 1). The PCR mixture was prepared in a total volume of 25 µL containing MgCl2 (2 mM), 10x PCR buffer (2.5 µL), dNTPs (200 mM), forward and reverse primers (400 µM), and Taq DNA polymerase (2 units) (CinnaGen, Iran). PCR cycling was carried out as publicized before (MIKI et al., 2008). After electrophoresing the amplified products in 1.5% agarose gel (Cinna Gen, Iran) and staining them with ethidium bromide, a UV light was used for photographing.

Amplification of netB gene. A pair of primers was used to detect the fragment (Table 1). The PCR mixture was prepared in a total volume of 25 µL containing MgCl2 (2.5 mM), 10x PCR buffer (2 µL), dNTPs (0.2 mM), forward and reverse primers (0.1 µM), and Taq DNA polymerase (2.5 units) (CinnaGen, Iran). Negative and positive controls were included in all PCR reactions. PCR cycling was carried out as publicized before (TOLOOE et al., 2011). After electrophoresing the amplified products in 1.5% agarose gel (Cinna Gen, Iran) and staining them with ethidium bromide, a UV light was used for photographing.

Amplification of tetracycline and enrofloxacin resistance genes. PCR assay was carried out to detect tetracycline (*tet*A) and enrofloxacin (*gyr*A) resistance genes using specific pairs of primers (Table 1). The PCR mixtures for both *tet*A and *gyr*A genes were prepared in a total volume of 25 µL, containing MgCl2 (1.5 mM), 10x PCR buffer (2.5 µL), dNTPs mixture (0.5 mM for *tet*A and 0.2 mM for *gyr*A genes), forward and reverse primers (0.3 µM for *tet*A and 0.1 mM for *gyr*A genes), and Taq DNA polymerase (2 units) (Cinna Gen, Iran). PCR cycling was carried out as explained in earlier publications (PAYOT et al., 2002; SCHWAIGER et al., 2010; WANG et al., 2010). After electrophoresing the amplified products in 1.5% agarose gel (Cinna Gen, Iran) and staining them with ethidium bromide, a UV light was used for photographing.

Tetracycline and enrofloxacin susceptibility test. The antimicrobial susceptibility test was carried out using the MIC (Minimal Inhibitory Concentration) value, which was determined using the broth microdilution technique according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). 100 µL of double TSB was initially added to each well of 96-well panels. 100 µL of each antibiotic of tetracycline (400 µg/mL) and enrofloxacin (100 µg/mL) was subsequently serially diluted

in the wells, and 10 µL of *C. perfringens* suspension (105 CFU/mL) was finally added to each well followed by incubation anaerobically at 37 °C for 24 h (the experiment was repeated in triplicate). The bacterial growth was determined by measuring the O.D. at 600 nm of each well (Bio Tek, USA). A *C. perfringens* strain was determined as a positive control, while TSB medium and antibiotics were considered as negative controls. The MIC was confirmed at the lowest dilution of an antibiotic component with visually complete inhibition of bacterial growth. The growth inhibition percentage of bacteria was reported using the equation as follows:

[(OD positive control (between 0 h to 24 h) - OD samples (between 0 h to 24 h)) / OD positive control (between 0 h to 24 h)] × 100 (KATHER et al., 2006).

Statistical analysis. To assess the statistically significant differences in the distribution of few anti-microbial resistance genes, a Chi-square contingency test was employed. A P-value <0.05 was considered significant.

Results

PCR assays of cpa, cpe and netB genes. Out of 200 swab samples, 90 and 70 suspected colonies were respectively isolated from cattle and sheep on the SPS agar. The suspected colonies were then subjected to the PCR, and of these 36 (40%) and 25 (35.7%) were identified as *C. perfringens* type A (*cpa*-positive) in cattle and sheep, respectively. In addition, from the type A isolates, 1 (2.7%) isolate was shown to carry both CPE and NetB in cattle, while no positive gene carriers were found in sheep (Table 2). 233 bp and 383 bp amplicons corresponded to *cpe* and *net*B genes respectively (Fig. 1).

Table 2. Percentage of *cpa*, *cpe* and *net*B genes and tetracycline and enrofloxacin (*tetA*, *gyrA*) resistance genes from *C. perfringens* type A

Carcass	Number of presumptive colonies	*cpa*-positive (type A)	type A- *cpe*	type A- *net*B	type A- *tet*A resistance	type A- *gyr*A resistance
Cattle	90	36 (40%)	1 (2.7%)	1 (2.7%)	24 (66.7%)	2 (5.55%)
Sheep	70	25 (35.7%)	0	0	13 (52%)	1 (4%)

Amplification of tetracycline and enrofloxacin resistance genes. The results revealed that 66.7% (24/36) and 52% (13/25) of the *C. perfringens* type A carried the *tet*A gene in cattle and sheep respectively. Moreover, 2 (5.55%) and 1 (4%) of the cattle and sheep isolates respectively, were resistant to the *gyr*A gene (Table 2). 888 bp and 582 bp amplicons respectively corresponded to the *tet*A and *gyr*A genes (Fig. 2).

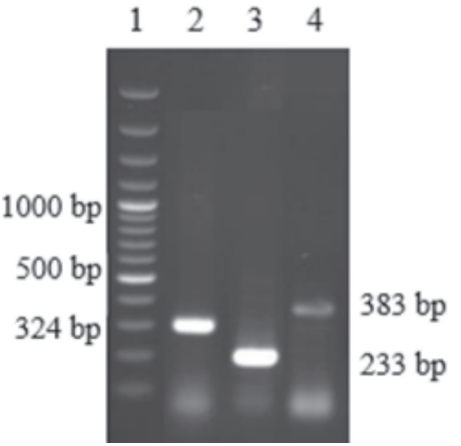

Fig 1. Representation of agarose gel electrophoresis for recognition of *cpa*, *cpe* and *net*B amplicons of *C. perfringens* using PCR reaction. Lane 1: 100 bp Marker, Lane 2, 3 and 4: 324 bp, 233 bp, and 383 bp positive samples, respectively.

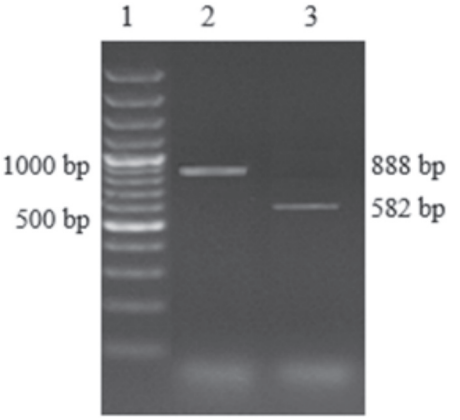

Fig 2. Representation of agarose gel electrophoresis for detection of *tet*A and *gyr*A resistance amplicons of *C. perfringens* using PCR reaction. Lane 1: 100 bp Marker, Lane 2 and 3: 888 bp and 582 bp positive samples respectively.

Tetracycline and enrofloxacin susceptibility test. The MIC values of tetracycline selected from *cpa*-positive *C. perfringens* isolates from cattle and sheep were determined in a concentration range of 3.12-25 µg/mL. Of the *tet*A-positive isolates, 45.8% (11/24) and 92.3% (12/13) cattle and sheep respectively were shown to have high-level resistance (MIC ≥16 µg/mL). Moderate resistant levels (4 < MIC <16 µg/mL) were also found in

41.6% (10/24) and 7.7% (1/13) cattle and sheep, respectively. Furthermore, there was a significant relationship between high or moderate tetracycline resistance and *tet*A positive isolates in comparison of the susceptible isolates ($P<0.05$).

The MIC value for enrofloxacin recorded in the concentration range of 0.0002-0.031 µg/mL for both animal species, indicated that all the *gyr*A positive isolates were susceptible to enrofloxacin (MIC ≤4 µg/mL) (Table 3).

Table 3. MIC values of tetracycline and enrofloxacin (*tetA*, *gyrA*) resistance genes of *C. perfringens* in resistant and susceptible isolates from cattle and sheep

	*tet*A (cattle)		*tet*A (sheep)		*gyr*A (cattle)		*gyr*A (sheep)	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
High-level resistance MIC ≥ 16 µg/mL	11	0	12	0	0	0	0	0
Moderate-level resistance 4 < MIC < 16 µg/mL	10	0	1	2	0	0	0	0
Susceptible MIC ≤ 4 µg/mL	3	12	0	10	0	36	0	25
Total	24	12	13	12	0	36	0	25

Discussion

CPE and NetB toxins are known to be the most important excretions produced by *C. perfringens* causing serious diseases in human and animals. Although *C. perfringens* is extensively distributed in the environment, just less than 5% of the pathogen strains carry the *cpe* gene (DAMME-JONGSTEN et al., 1989; KOKAI-KUN et al., 1994). About 40 outbreaks of *C. perfringens* foodborne GI diseases were recorded during 2000 to 2005 in Japan, and approximately 4000 people are estimated to become infected annually (MIKI et al., 2008). We found that the cattle and sheep carcasses slaughtered in Shiraz, Iran, were contaminated with *C. perfringens* type A at the rate of 40% and 35.7%, respectively. Several studies have been conducted to determine the prevalence rate of *cpe* and *net*B genes isolated from various sources, especially raw meat and poultry (WEN and McCLANE 2004). For instance, the occurrence of the *net*B gene isolated from chickens and cattle was reported as 9.2% and 1.4%, respectively (MARTIN and SMYTH 2009). In American retail markets, *C. perfringens* was identified in 21% and 38% of beef and lamb respectively, and one was *cpe*-positive (WEN and McCLANE 2004). The incidence of

C. perfringens isolates from slaughtered beef and lamb was found to be 29% and 85%, respectively (SMART et al., 1979). In Japan, the results demonstrated that 45.7% and 33.3% of Japanese retail raw beef samples tested were contaminated with *C. perfringens* bacteria, and approximately 2% were *cpe*-positive (MIKI et al., 2008). A study carried out on the presence of *C. perfringens* in minced meat from the north-east of Iran showed that 12.5% of samples were contaminated with this pathogen, of which 18% carried *cpa* gene toxins. In addition, the rate of *C. perfringens* type A was 29.03% in broiler meat (AFSHARI et al., 2015a; AFSHARI et al., 2015b). Based on the previous studies, the variations in the prevalence of *C. perfringens* were probably associated with factors including the method of slaughter, the overall sanitation condition, the total number of samples, and the methods chosen to detect the bacteria.

Nowadays, antibiotic resistance is rising significantly in the developing world where antimicrobial drugs are utilized without restrictions, and as such this issue has attracted a great deal of concern (JEAN and HSUEH 2011). *C. perfringens,* as a microflora of the human and animal GI tracts, plays a crucial role in antibiotic resistance. Isolates taken from patients with antibiotic-associated diarrhea and sporadic diarrhea were considered as plasmid-borne *cpe* (MIKI et al., 2008; TANSUPHASIRI et al., 2005). Various reports have been published about the resistance to multiple antibiotics of *C. perfringens* strains in humans and animals (KATHER et al., 2006). According to this study, 66.7% and 52% of the *C. perfringens* type A isolates were tetracycline-resistant, and 5.55% and 4% were enrofloxacin-resistant, in cattle and sheep carcasses, respectively. The MIC values also showed high level tetracycline resistance for both cattle and sheep. Antimicrobial resistance to *C. perfringens,* isolated from various sources in Thailand, revealed that the highest resistance was attributed to tetracycline (56.2%) using the agar dilution method (TANSUPHASIRI et al., 2005). Earlier studies reported that tetracycline resistance is the most common antimicrobial resistance (CHOPRA and ROBERTS 2001). KATHER et al. (2006) documented that 96% of the *C. perfringens* isolates were identified as *tet*A gene positive, while 22.5% of *C. perfringens* isolates were resistant to tetracycline, using the MIC technique. These results support our findings.

Even though the reservoirs of *C. perfringens* food poisoning are partially recognized, as this bacterium is naturally present in feces and it is actually the causative agent of human food poisoning and diarrhea, it seems that contamination is likely due to the poor handling of the carcasses, specifically at the time of slaughtering. We suggest that special attention should be paid to the evisceration process, and the spread of the feces onto the whole carcass throughout this task should also be avoided.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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SAŽETAK

Trovanje hranom uzrokovano bakterijom *Clostridium perfringens* tipa A često uzrokuje *C. perfringens* enterotoksin (CPE), kodiran kromosomskim *cpe*. Kontaminacija mesa s *C. perfringens* obično uzrokuje otrovanje hranom. Da bismo doznali više informacija o uzročniku, iz trupova goveda i ovaca zaklanih u klaonicama Shiraza, identificirali smo tip A koji sadržava gene *cpe* i *netB*. Osim toga, u izoliranim bakterijama *C. perfringens* utvrdili smo prevalenciju plazmida rezistentnih na antibiotike. Obriskom cijele vanjske i unutarnje površine nasumično odabranih trupova, prikupljeno je 200 uzoraka koji su obrađeni selektivnom kulturom na sulfadiazin-polimiksin sulfatnom agaru (SPS). Sumnjive kolonije dodatno su identificirane primjenom specifičnih početnica kako bi se potvrdila prisutnost gena *cpa*, *cpe*, *netB* te gena za otpornost na tetraciklin i enrofloksacin. Naši su rezultati pokazali da je PCR analizom od 90, odnosno 70 kolonija pozitivnih kultura iz uzoraka goveda i ovaca, njih 40 %, odnosno 35,7 % identificirano kao *C. perfringens* tipa A. Štoviše, iz izolata tipa A dobivenih od goveđih trupova, samo je jedan izolat (2,7 %) bio pozitivan i za gene *cpe* i za *netB*. MIC vrijednosti također su pokazale visoku razinu otpornosti na tetracikline kod goveda (45,8 %) i ovaca (92,3 %), dok su svi PCR pozitivni na *C. perfringens* tipa A bili osjetljivi na enrofloksacin. Visoka prevalencija bakterije *C. perfringens* kod zaklanih životinja zajedno s visokom stopom otpornosti na tetraciklin upućuje na potrebu za oprezom u primjeni antibiotika kod životinja koje služe za ljudsku hranu.

Ključne riječi: *Clostridium perfringens;* genomi *cpe* i *netB*; otpornost na antibiotike