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Detection and Frequency of Enterotoxin (*cpa*, *cpe*) Genes of *Clostridium perfringens* Isolated from Dehydrated Vegetables by PCR

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ABSTRACT

Background & Objective: *Clostridium perfringens* is an anaerobic bacterium, commonly present in retail foods. Its enterotoxin-producing ability, short generation time, ability to grow at elevated temperatures, and spore-forming ability allow *C. perfringens* to survive in food-processing temperatures, and cause foodborne illness. The aim of study was to screen dehydrated vegetables contaminated with *Clostridium perfringens enterotoxin (CPE)* and *C. perfringens alpha-toxin (CPA)*.

Materials & Methods: This descriptive-analytical study was carried out on 140 samples (70 unpacked and 70 packed). The samples included dehydrated vegetables collected from different areas of Tehran, Iran. Samples were inoculated on peptone and sulfite polymyxin sulfadiazine (SPS) agar for enrichment. The enrichment culture was then incubated on anaerobic condition for 48 hours. The black colonies were selected for identification test and polymerase chain reaction (PCR). The bacterial colonies were identified by biochemical tests, and duplex PCR was performed for *CPE* and *CPA* genes.

Results: In general, 13 samples (9.3%) were identified as contaminated with *C. perfringens* using phenotypic methods; all of the isolates were also positive for *CPA,* but negative for *CPE* gene. The contamination rate for packed and unpacked vegetables was 12.8% and 5.7%, respectively.

Conclusion: Our findings showed that contamination of packed samples was higher than unpacked ones, which might be due to drying as well as packaging process. We found that these isolates were negative for enterotoxin.

Keywords: *Clostridium perfringens*, Dehydrated vegetables, Enterotoxin

Introduction

Clostridium perfringens ranks among the most widespread bacteria, with an ubiquitous environmental distribution in soil, sewage, food, feces, and the normal intestinal flora of humans and animals (1, 2). According to the Center for Disease Control and Prevention (CDC) estimation, more than one million people are infected by *C. perfringens* each year (3). Food poisoning can be caused by *C. perfringens enterotoxin (CPE)* produced by *C. perfringens* spores in the small intestine, which can germinate in foods such as meat and poultry. Main symptoms of the disease are nausea, abdominal pain, and diarrhea. The disease is usually mild and self-limiting in healthy individuals, with symptoms resolving within 24 hours (4,5). The *CPE* is encoded by its gene located on the chromosome or plasmid of the bacterium (6-8). Expression of the *CPE* is an important determinant of *C. perfringens* causing food poisoning (9). Available diagnostic tests for the toxin detection are immunoenzymatic assays including latex agglutination, immunodiffusion, immunoelectrophoresis , ELISA, and Western blot. All these tests depend on high quantity of the enterotoxin in samples. Some strains may carry a silent *CPE* gene, resulting in missed identification of toxigenic strains. One of the reliable and useful methods for the detection of *C. perfringens* toxins is polymerase chain reaction (PCR). This method does not depend on enterotoxin concentration (10). Many of *C. perfringens* food poisoning events occur due to the consumption of

contaminated meat and poultry products (11). Detection of toxigenic *C. perfringens* is important because spore forming bacteria are Stable in the environment. The dehydrated vegetables are routinely used in Iran in various foods, including main dishes and herbal medicines. The aim of this study was to isolate and identify *C. perfringens* in dehydrated vegetables in Iran.

Materials and Methods

Sampling

A total of 140 samples of dehydrated green leafy vegetables, including dill, parsley, coriander, tarragon, mint, and mixed vegetables (crumb and soup) were collected from different areas of Tehran, Iran. Samples were collected as unpacked dehydrated vegetables (*n*=70) and packed (*n*=70). The study was approved by the Ethics Committee of Tehran University of Medical Sciences (code=IR.TUMS.SPH.REC.1394.775).

Culture and Isolation

To evaluate the samples, 10 g of dehydrated vegetables was diluted in 90 mL of sterile 0.1% peptone water, then 10 mL of this suspension was added to 10 mL of thioglycollate medium. Two thioglycollate tubes were prepared from each sample, one tube was incubated at 75°C for 30 min for the detection of *C. perfringens* spores and the second tube was incubated at 37°C in an anaerobic jar with Anaerocult A for 48 h. After enrichment step, the supernatant was discarded, and two drops of the pellets were streaked on sulfite polymyxin sulfadiazine (SPS) agar plates and incubated at 37° C for 24-48 h in an anaerobic jar. Black colonies on SPS agar were subjected to Gram staining and biochemical tests.

Biochemical Tests

Black colonies were assessed for nitrate reduction, gelatinase, production of double zone, motility test, lecithinase production in egg yolk agar, and acid production by lactose and glucose fermentation. The isolates confirmed as *C. perfringens* were stored at -20°C.

PCR

For genomic DNA extraction, 5-6 colonies grown on SPS agar were inoculated in 200 µl of sterile double distilled water and boiled at 100˚C for 5-10 min. Following the centrifugation at 12,000 g for 3 min, the supernatant was used as the template DNA for PCR assay (12, 13). The specific primers were used to amplify 324 and 233 base pair (bp) of *C. perfringens alpha-toxin (CPA)* and *CPE* genes, respectively (Table1). Reference strain of *C. perfringens* strain CIP 106157 was used as a positive control for detection of *CPA* and *CPE* genes. The PCR was performed in a total volume of 20 µL containing 10 µL of master mix (Pishgam, Iran), 10 ng of DNA, 8.5 µL of H_2O, and 0.25 mM of each primer (Bioneer, Seoul, South Korea). Thermo cycler (Peqlab, Germany) was set with the following conditions: initial denaturation at 95°C for 5 min and 30 cycles including denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 3 min, and final extension at 72°C for 4 min (14). Electrophoresis was performed in 1% agarose gels (Invitrogen, USA) and gel was stained with 0.5 µg ml^{-1} ethidium bromide (Sigma, USA).

Nucleotide Accession Number

The partial sequence of *CPA* gene was deposited in GenBank under the accession number of KU166870.

Table 1. Specific primer sequences targeting *cpa* and *cpe*

Gene	Sequence(5′-3′)	Amplicon size (bp)	Reference
Cpa	GCTAATGTTACTGCCGTTGA CC TCTGATACATCGTGTAA	324	(9)
Cpe	GGAGATGGTTGGATATTAGG GGACCAGCAG TTGTAGATA	233	(9)

Results

Out of 140 samples, 13 (9.3%) were identified as contaminated with *C. perfringens*. Among the contaminated samples, 9 (12.8%) and 4 (5.7%) samples were from packed and unpacked samples, respectively. Based on dried vegetables breeds showed no contaminations in soap vegetables, while 1 (0.7%) parsley, 2 (1.4%) coriander, 2 (1.4%) mint, 2 (1.4%) tarragon, 3 (2.2%) dill, and 3 (2.1%) vegetable crumble samples were contaminated with *C. perfringens*. Using duplex PCR, all isolates were positive for *CPA* gene while all were negative for *CPE* gene (Figure 1). This finding indicated that the rate of *C. perfringens* was 12.8% on the packed dehydrated vegetables and 5.7% on unpacked vegetables.

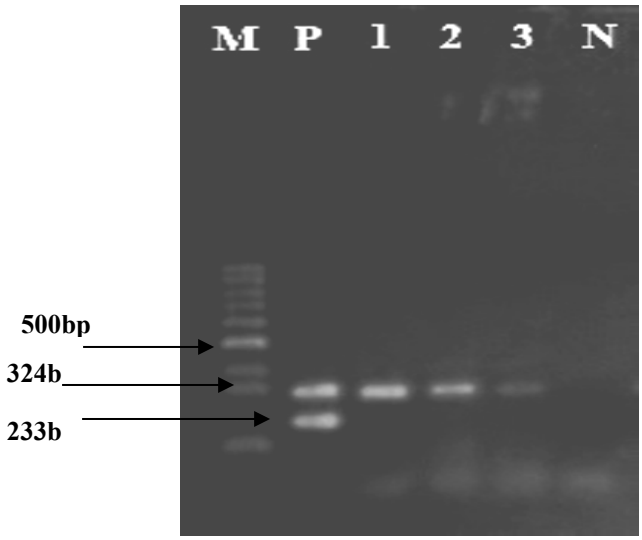

Figure1. Duplex PCR for cpa and cpe genes. M: marker 100bp, P: CIP106157 positive control for cpa and cpe genes. N: negative control. Line

Discussion

Our results showed that there was no relationship between the positive rate of *C. perfringens* and brand or breed of the vegetables. Although there are many studies on typing and toxin detection of *C. perfringens*, none of them has investigated the contamination of dried vegetables so far. Dried vegetables are usually used as cooked or undercooked; thus, consumption of these products may result in food illness. In addition, if the products are contaminated with spores, the stability of spore to heat is another problem **(15)**.

Despite the low frequency of *CPE* positive and *C. perfringens* in foods especially in meat, poultry, and dried vegetables, these products can be sources of toxins. Various reports showed that the rate of *C. perfringens*-associated food poisoning varies around the world **(11,16, 17)**. Unfortunately, there is no comprehensive data showing the frequency of *C. perfringens* in food poisoning in Iran. This could be due to various reasons including difficulty in registration of patient data or short period of symptoms (24 h). In this study, we determined that 9.3% of dehydrated vegetables were contaminated with *C. perfringens*. This contamination might be due to the presence of spores in dehydrated samples. The spores are resistant to desiccation, heating, and other conditions. In 2009, Sagoo *et al.* reported that *C. perfringens* contamination in dried vegetables and spices is 0.4%. In some other studies conducted in the UK (1975, 1985, 1986), the isolation rate of *C. perfringens* isolation from dried vegetables was 0 to 7.6% **(15)**.

The frequency of *C. perfringens* isolated from packed vegetables was more than unpacked ones. This finding may be related to inappropriate processing during packaging or lack of hygiene consideration in factory, transportation, and packaging. The isolates were investigated for *CPA* and *CPE* genes. Duplex PCR was applied for 13 confirmed isolates, in which all samples were positive for *CPA* gene and negative for *CPE* gene. As far as the researchers investigated, no study has been carried out on dehydrated vegetables in Iran yet. But some studies have been carried out in Iran on isolation of *C. perfringens* from poultry meats. Poursoltani *et al.* (in 2014) detected six isolates from 180 packed poultry samples. They used multiplex PCR for *C. perfringens* subtyping and single PCR for *netB* and *tpel* genes in poultry with necrotic enteritis symptoms **(18)**. In Iran, Zandi *et al.* (in 2014) showed that 100% ostrich dung samples were positive for *CPA* gene. The multiplex PCR method was used that have not been reported in Iran **(19)**. The difference in frequency of contamination may be due to different contamination sources. Erol *et al.* (2008) used multiplex PCR on ostrich meat, and showed that 12.2% (22/180) isolates were positive for *CPA* gene but negative for *CPE* gene **(12)**.

Conclusion

Our results showed that 13 (9.3%) samples were contaminated with *C. perfringens*. *H*owever, the bacteria were negative for *CPE*. The higher contamination rates in packed vegetables compared to unpacked ones might indicate the lack of suitable hygienic considerations on drying and packaging processes.

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Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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This research resulted from an independent research without receiving any financial support.

Conflict of Interest

Authors declared no conflict of interest.

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