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ORIGINAL ARTICLE

The Prevalence of *Clostridium difficile* and *Clostridium perfringens* in Minced and Ground Beef in Iran

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KEYWORDS

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ABSTRACT: In this study, due to the importance of human societies, the prevalence of *Clostridium difficile* and *Clostridium perfringens* in minced and ground beef was investigated in Qazvin city, Iran. All samples were collected randomly by sampling method. The number of samples taken was estimated based on statistical methods and according to previous studies. *Clostridium difficile* moxalactam norfloxacin (CDMN) culture media was used to isolate *Clostridium difficile* and TCS Agar and TPGY culture media was used for *Clostridium perfringens*. After isolation, a PCR (Polymerase Chain Reaction) test was used to confirm the species diagnosis. According to the results, 21.26% of all samples were infected with these two bacteria. The prevalence of *Clostridium perfringens* (18.04%) was significantly ($p < 0.05$) higher than Clostridium *difficile* (3.22%). Given the results and the pathogenicity of *Clostridium* species, especially *Clostridium difficile* and *Clostridium perfringens*, special attention should be paid to the methods reducing the contamination of these pathogenic bacteria in raw food.

INTRODUCTION

Clostridia are anaerobic, gram-positive, and spore-producing bacilli important for pathogenesis, especially food-borne diseases [1]. *Clostridia* are widely distributed in nature and they can be considered a food-borne pathogen important to public health because of their abilities to produce spores, grow very rapidly in food, and produce toxins. *Clostridia* are found everywhere in the environment, including in the soil (as the main source), the bodies of animals and humans, plants and vegetables. Therefore, due to their presence in the environment, the probability of disease in humans and animals is very high [2, 3].

The pathogenic potential of *Clostridium perfringens* is attributed to their ability to produce at least 29 types of toxins and enzymes. *C. perfringens* are classified into five different types (A, B, C, D, and E) based on their ability to produce four major deadly toxins, including alpha, beta, iota, and epsilon [4, 5]. *C. perfringens* require various amino acids for growth and survival, which are essential for

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bacteria. Therefore, high-protein foods such as meat provide a very nutritious environment for bacteria [6].

The disease by *C. perfringens* usually manifests itself with abdominal pain, nausea, and diarrhea 6–24 hours after the consumption of contaminated food. In the vast majority of cases, it is self-limiting; but in the vulnerable population (elderly, very young patients), deaths can occur due to dehydration [3, 7].

Clostridium difficile was introduced in 1970 as a gastrointestinal pathogen. Due to the high strength of toxin production and its resistance to various environmental conditions, this bacterium is one of the most dangerous pathogens, especially intestinal diseases [8]. *C. difficile* produces two types of toxins: enterotoxin and cytotoxin [9]. Moreover, *C. difficile* can be isolated from various animals, including food animals like pork, beef, and turkey. Those isolated from food, especially raw meat, are very importance in public health. They are very important for concerns about the transmission of the disease to humans [10].

According to various sources, as well as reports from related organizations, the presence of *C. perfringens* and *C. difficile* in food is very important from the perspective of public health and human and animal diseases. In Iran, a few researches have been done on the abundance of these two bacteria in food. In Qazvin city (hot-summer Mediterranean climate; altitude, 1,278 m; longitude, 50°00′E, and latitude, 36° 16′N), the prevalence and frequency of these two bacteria in food has not been studied so far. Therefore, in this study, the prevalence and frequency of *C. perfringens* and *C. difficile* in ground and minced raw meat at the city's butchers were investigated.

MATERIALS AND METHODS

Sampling and isolation of C. difficile

Ninety-three samples, including 42 pieces of ground and 51 minced meat samples from Qazvin city (from three parts of the north, south, and center of the city) were collected by random sampling method using a list prepared from meat supply centers (butchers). All samples were transferred to the laboratory of Qazvin University of Medical Sciences in sterile conditions and tested on the day of sampling.

First, the samples were placed one by one in the stomacher and completely homogenized. For isolation, enrichment was performed by transferring 5 ml of each sample to 25ml of the Clostridium difficile moxalactam norfloxacin selected as the liquid media (Merck, Germany). In anaerobic conditions, the samples were placed in an incubator at 37°C. After 7 days, the samples were removed and given an alcohol shock (transferred to two ml of liquid to two ml of ethanol and stored at ambient temperature for 60 minutes). After the alcohol shock, the samples were centrifuged at 3000 rpm for 10 minutes. The sediment at the bottom of the tube was planted on a CDMN agar media containing 7% of sheep blood and incubated under anaerobic conditions at 37˚C for 72 hours. Suspicious strains were cultured on the blood agar media and anaerobic incubation was performed at 37˚C for 48 hours. Finally, biochemical diagnosis was made by shape, odor, and gram staining on positive strains.

Sampling and isolation of C. perfringens

A hundred-thirty-three samples, including 81 pieces of ground and 52 minced meat samples from Qazvin city (from three parts of the north, south, and center of the city) were collected by random sampling method using a list prepared from meat supply centers (butchers). All preparation steps were similar *to C. difficile* bacteria.

The TPGY media (Merck, Germany) was used for enrichment. The samples were cultured in TSC agar culture media (Merck, Germany) after a heat shock. Eventually, the suspected strains were confirmed by catalase testing and gram staining.

DNA extraction

The isolated bacteria were cultured on Brain Heart Infusion Broth media and incubated at 37˚C for 24 hours. Subsequently, the enzyme lysozyme was added to the samples (30min) and the extraction of genomic DNA was performed by bowling method [11]. Finally, the purity and

absence of contamination in the extracted DNA sample were measured and confirmed.

PCR

The primers used in this study were based on the *TcD* gene sequence used using the Primer-BLAST software and the NCBI (National Center for Biotechnology Information) gene bank. The designed primer to detect *C. difficile* and *C. perfringens* is shown in Table 1 [11].

Table 1. Primer nucleotide sequence

Gene	Nucleotide sequence	Size
TcD	F-GGCTGAAGCTAATGCAGATAATG R- CCTCTCTCTGAACTTCTTGCTAAT	421 pb

After preparation of specific primers as well as 10-pmol concentration, PCR reaction was performed in a volume of 20 µl. To perform the PCR reaction, 10 µm MasterMix, 1 µm Primer, 3 µl extracted DNA, and 6 µl distilled water were used. Finally, PCR analysis was performed on 1% agarose gel and TBE buffer and specific bands were evaluated with Transilluminator.

Statistical analysis

SPSS software version 25 and the Chi-square with a significant level of $p < 0.05$ were used for statistical analysis of results.

RESULTS AND DISCUSSION

Figures 1 and 2 show the result of a molecular study of *C. difficile* and *C. perfringens*, respectively.

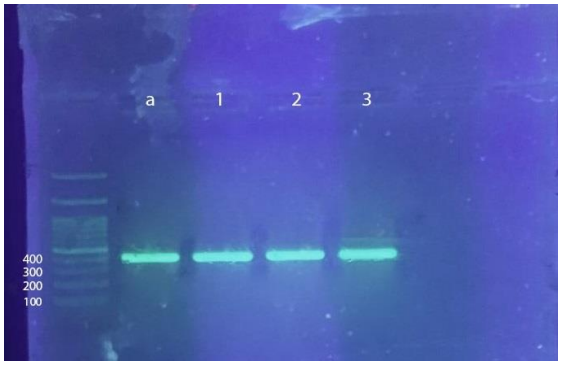

Fig. 1. Gel electrophoresis of PCR products of *C. difficile* samples
(First Lane, marker 100-1000 bp; Lane a, control; Lane 1-3, *C. difficile* in samples)

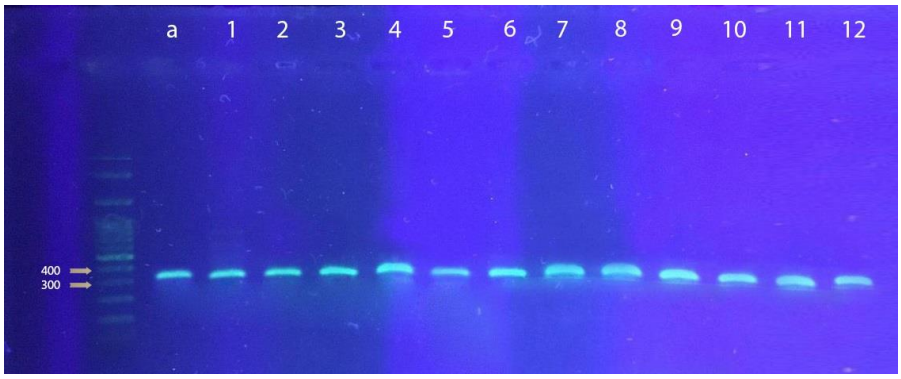

Fig. 2. Gel electrophoresis of PCR products of *C. perfringens* samples
(First Lane, marker 100-1000 bp; Lane a, control; Lane 1-12, *C. perfringens* in samples)

The results of the prevalence of *C. difficile* have been shown in Table 2. As observed in Table 2, the amount of contamination in ground meat is higher than that in minced meat. Overall, 3.22% of the collected samples were positive for *C. difficile*.

Table 2. Frequency and prevalence of *C. difficile*

Sample	Total sample number	Number of positive samples	Frequency
Minced	42	1	1.07%
Ground	51	2	2.15%
Total	93	3	3.22%

There have been several studies in Iran and around the world on the high levels of *C. difficile* in food and meat. Esfandiari et al. investigated the prevalence of C. difficile in hamburgers and meat processing plants in Isfahan province, Iran. Their results showed that 4.2% of the samples (211) were positive for bacteria [11]. Nayebpour and Rahimi studied the prevalence of *C. difficile* in a variety of edible oysters sampled from Bushehr province, in southern Iran. Their results revealed that 3.71% of oysters were positive for bacteria [12]. There have been various studies around the world regarding the presence of *C. difficile* in raw seafood. For example, Pasquale et al. described that 26 out of 53 (49%) of edible bivalve molluscs, particularly *Mytilus galloprovincialis* (48%) and *Tapes philippinarum* (53%), caught from Italy were contaminated with *C. difficile* [13].

Esfandiari et al. investigated the prevalence of *C. difficile* in raw beef in Isfahan, Iran. Twelve samples out of the total samples tested by them (100 samples) were reported to be positive for bacteria (12%). Among the 12 positive samples, four samples were minced meat and eight samples were ground meat [14]. Despite the large number of positive samples, more positive bacterial samples in ground meat than in the minced ones was in line with the results of the present study. In various studies, positive samples could be due to contamination of the meat grinder more, requiring further study.

In a study conducted in the Netherlands, the rate of contamination in lamb meat with *C. difficile* was 6.3% that is higher than the total amount of contamination in this study [15]. In another study performed in Sweden, the rate of infection with *C. difficile* in ground meat was 2.04%, almost the same as the results of our study [16].

The results of the prevalence of *C. perfringens* have been shown in Table 3. According to the results, the amount of contamination in ground meat is higher than that in minced meat like *C. difficile*. The prevalence and the number of positive samples in samples containing *C. perfringens* are significantly more than *C. Difficile* ($p < 0.05$).

Table 3. Frequency and prevalence of *C. perfringens*

Sample	Total sample number	Number of positive samples	Frequency
Minced	52	10	7.51%
Ground	81	14	10.52%
Total	133	24	18.04%

The results of various studies conducted in several regions of Iran, including Khuzestan and Tabriz, show that *C. perfringens* with *C. difficile* are the most important pathogenic species of *Clostridia* with food origin [17]. Furthermore, there have been several studies of the prevalence of *C. perfringens*, leading to disease in human societies, the most recent of which occurred in Greece in 2019 [18]. In another study in Diyarbakir province, Turkey,

96% of ground beef samples in supermarkets and butchers were contaminated with *C. perfringens* [19]. A new study examines the risk of spreading *C. perfringens* in Cornish pasties in the UK. The results of their study revealed that the contamination of raw materials such as meat and spices is one of the food contamination sources. Therefore, managing and reducing the burden of pollution in raw materials will decline the total pollution in the consumed food and thus reduce the risk of disease [20].

Table 4 shows the prevalence and frequency of positive bacterial samples in three sampling areas in the city. According to the results, the amount of positive results for both bacteria in the collected samples from the south of city is significantly higher than other areas ($p < 0.05$). As observed in Table 4, the division of the three sampling areas contains significant results. The total positive results for the two bacteria in the samples collected from the north of the city are significantly lower than the south and center of the city, especially *C. difficile*, not isolated from any of the samples in the north of the city.

Table 4. Prevalence and frequency of positive bacterial samples in three sampling areas

Bacteria	South of city			North of city			Center of city		
	Total number	Total positive sample	Frequency	Total number	Total positive sample	Frequency	Total number	Total positive sample	Frequency
C. perfringens	45	18	40%	44	2	4.54%	44	4	9.09%
C. difficile	31	2	6.45%	31	0	0	31	1	3.22%
Total	76	20	46.45%	75	2	4.54%	75	5	12.31%

According to the results of the present study, both *C. difficile* and *C. perfringens* bacteria are present in minced and ground meat in Qazvin city (Iran), which can transmit the disease and infection. Since the number of positive samples is higher in ground meat samples, it is recommended to do more studies on transmission routes (meat grinder, staff hand, etc.) as well as methods to reduce the frequency of bacteria.

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Conflict of interests

The authors declare that there are no competing interests associated with the manuscript.

REFERENCES

1. Borriello S.P., Aktories K., 2005. *Clostridium perfringens*, *Clostridium difficile*, and other *Clostridium* species. In: Borriell SP, Murray PR, Funke G, editor. Topley and Wilson's microbiology and microbial infections. 10th ed. London: ASM press. pp. 1098-1136.

2. Candel-Pérez C., Ros-Berruezo G., Martínez-Graciá C., 2019. A review of *Clostridioides* [*Clostridium*] *difficile* occurrence through the food chain. Food Microbiol. 77, 118-129.

3. Lindström M., Heikinheimo A., Lahti P., Korkeala H., 2011. Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. Food Microbiol. 28, 192–198.

4. Novak J.S., Juneja V.K., 2002. *Clostridium perfringens*: hazards in new generation foods. Innov Food Sci Emerg Technol. 3, 127-132.

5. Alphons J., Cornelia W., Dirk J., 2009. A multiplex PCR for toxin typing of *Clostridium perfringens* isolates. Vet Microbiol. 136, 411-412.

6. Adams M.R., Moss M.O., 2006. Food microbiology (2nd ed.). Cambridge: The Royal Society of Chemistry.

7. Brynestad S., Granum P.E., 2002. *Clostridium perfringens* and foodborne infections. Int J Food Microbiol. 74, 195–202.

8. Gould L.H., Limbago B., 2010. *Clostridium difficile* in food and domestic animals: a new foodborne pathogen? Clin Infect Dis. 51(5), 577-582.

9. Hookman P., Barkin J.S., 2007. Reviwe: *Clostridium difficile*-associated disorders/diarrhea and *Clostridium difficile* colitis: the emergence of a more virulent era. Digest Dis Sci. 52, 1071-1075.

10. Weese J.S., Reid-Smith R.J., Avery B.P., Rousseau J., 2010. Detection and characterization of *Clostridium difficile* in retail chicken. Lett Appl Microbiol. 50, 362–365.

11. Esfandiari Z., Weese S., Ezzatpanah H., Jalali M., Chamani, M., 2014. Occurrence of *Clostridium difficile* in seasoned hamburgers and seven processing plants in Iran. BMC Microbiol. 14, 283(1-7). https:// doi.org/ 10.1186/ s12866-014-0283-6

12. Nayebpour F., Rahimi E., 2018. Prevalence, antibiotic resistance, and toxigenic gene profile of the *Clostridium difficile* isolated from molluscan shellfish. J Food Saf. e12586. https://doi.org/10.1111/jfs.12586

13. Pasquale V., Romano V., Rupnik M., Capuano F., Bove D., Aliberti F., Dumontet S., 2012. Occurrence of toxigenic *Clostridium difficile* in edible bivalve molluscs. Food Microbiol. 31(2), 309–312.

14. Esfandiari Z., Jalali M., Ezzatpanah H., Weese S., Chamani M., 2014. Examination of *Clostridium difficile* Contamination in beef meat distributed in Isfahan using culture and Multiplex-PCR method. Biological Journal of Microorganism. 7(8), 109-116. doi: 10.5812/jjm.16771.

15. Rupnik M., Songer J.G., 2010. *Clostridium difficile*: Its potential as a source of foodborne disease. Adv Nutr. 60, 53-66.

16. Von Abercron S.M.M., Karlsson F., TrowaldWigh G., Wierup M., Krovacek K., 2009. Low occurrence of *Clostridium difficile* in retail ground meat in Sweden. J Food Prot. 72(8), 1732-1734.

17. Khademi F., Sahebkar A., 2019. The prevalence of antibiotic-resistant Clostridium species in Iran: a meta-analysis. Pathog Glob Health. 113(2), 58-66.

18. Mellou k., Kyritsi M., Chrysostomou A., Sideroglou T., Georgakopoulou T., Hadjichristodoulou C., 2019. *Clostridium perfringens* Foodborne Outbreak during an Athletic Event in Northern Greece, June 2019. Int J Environ Res Public Health. 16(20), 3967(1-5). https://doi.org/10.3390/ijerph16203967

19. Guran H.S., Vural A., Erkan M.E., 2014. The prevalence and molecular typing of *Clostridium perfringens* in ground beef and sheep meats. J Verbr Lebensm. 9, 121–128.

20. Gkogka E., Reija M.W., Gorris L.G.M., Zwietering M.H., 2019. Risk assessment of *Clostridium perfringens* in Cornish pasties in the UK. Food Control. 108, 106822(1-14). https://doi.org/10.1016/j.foodcont.2019.106822