Occurrence of *Clostridium difficile* in seasoned hamburgers and seven processing plants in Iran

Zahra Esfandiari1, Scott Weese2, Hamid Ezzatpanah1, Mohammad Jalali2* and Mohammad Chamani3

**Abstract**

**Background:** The recent increment of the incidence of Community Associated *Clostridium difficile* Infection (CA)-CDI has led to speculation that this disease is associated to foodborne transmission. Therefore it is critical to establish the community sources of CDI in order to implement the appropriate interventions. The present study was conducted to evaluate the prevalence of *C. difficile* in seasoned hamburger and examine the sources of *C. difficile* dispersal in hamburger processing plants. A total of 211 samples including hamburger ingredients, the final product, processing equipment and food contact surfaces were collected from seven hamburger processing plants to evaluate the routes of dispersal of *C. difficile*. The samples were assessed for the occurrence of *C. difficile* using culture and polymerase chain reaction (PCR) methods. All isolates were screened for the existence of toxin A, B and binary toxin genes. In addition, isolates were subjected to PCR ribotyping.

**Results:** Overall, 9/211 (4.2%) samples were positive. Toxigenic *C. difficile* were detected from 2/7 (28.5%) hamburger processing plants, in (3/54) 5.6% of beef meat samples, (2/56) 3.5% of swabs taken from the environment and (4/56) 7.1% of hamburger samples after both molding and freezing. *C. difficile* was not found in 45 non-meat ingredients including 14 defrosted onions, 14 textured soy proteins and 17 seasonings. All isolates contained tcdB gene while 7 strains were positive for tcdA and two remaining strains were negative for tcdA. None of the isolates harbored binary toxin gene (cdtB). PCR ribotyping of 9 isolates categorized into four ribotypes (IR21, IR 22, IR 23 and IR24). Ribotype IR 22 was the most common type 6/9 (66.6%) found. This genotype was isolated from raw meat, environmental samples and hamburger after both forming and freezing in one processing plant, suggesting raw beef meat as a possible major source of contamination.

**Conclusions:** Hyper-virulent strains of ribotype were not found in this study however, occurrence of other toxigenic strains indicate the public health significance of contamination of this product.

**Keywords:** *Clostridium difficile*, Meat, Processing plants, Ribotyping
C. difficile at the farm or retail level, and only a few surveys have been performed at meat processing plants [6,7,11-13]. Rodriguez-Palacios et al (2007) reported the presence of C. difficile up to 20% in unseasoned hamburgers in Canada [3]. Further studies indicated that C. difficile spores are relatively unaffected by processing such as freezing, refrigeration and cooking ([14-16]). Furthermore, the epidemiology and prevalence of C. difficile in food in Iran are limited, despite the importance of CDI and a previous study that found ribotype 078 to be a leading cause of CDI in people at one hospital in Isfahan, Iran [17]. The rate of CA-CDI reported to be 24% in Iran, with no data available on sources of infection [17]. Therefore, the objective of the present study was to determine the prevalence of C. difficile in seasoned hamburger and examine the sources of C. difficile dispersal in hamburger processing plants in the same location in Iran.

Methods
Sampling design
This study was conducted in Isfahan, central part of Iran (subtropical zone; mean long time rainfall, 120 mm; mean long time temperature, 33°C and 17.6°C in summer and autumn; altitude, 1,555 m; longitude, 51°30′ E; and latitude, 32°31′ N). Samples were taken from seven (coded A to G) available hamburger processing plants that supply products nationwide from July to December 2012. Samples collected during four visits (two visits in summer and two visits in autumn). Three of seven processing plants (coded A, B and G) were Hazard Analysis Critical Control Points (HACCP) certified. However, all processing plants were ranked in terms of Quality Assurance Managements (QA) and implementation of Good Manufacturing Practice (GMP) by the Ministry of Health (Category I, II and II). The hamburger patties consisted of 35% texturized soy protein, 30% beef meat, 16% onion, 9% frying oil, 8% wheat flour, 1.7% salt and 0.3% irradiated seasonings. The ground product was molded, sandwiched between two waxed paper sheets, packaged and frozen at −18°C for 24 h (Figure 1). Samples were collected based on their production day per week (one or two day production per week). At the hamburger processing plants, the ingredients had a different expiry dates and the irradiated seasonings, texturized soy protein and frozen onion, kept for longer time (for more than a few months). Therefore these products sampled less frequently than the beef meat (normally supplied on weekly bases) as a main ingredient of hamburger.

A total of 211 samples were obtained including: beef meat (n = 54), textured soy protein (n = 14) irradiated seasoning (n = 17), defrosted onion (n = 14), hamburger

Figure 1 Flow diagram for the production of hamburgers with sampling points (red diamonds for ingredient/final product and red circles for swab samples). *Packed boneless meat imported from Brazil. **Carcass meat provided from slaughterhouses in Iran (Number of positive samples for C. difficile in total sample is mentioned in parenthesis in related box).
patties after molding (n = 28) and hamburger patties after freezing (n = 28). Approximately 5-10 gram of each sample were collected aseptically in sterile bottles and transported in an insulated cold box to the laboratory. In addition, 56 environmental samples were taken from meat grinder and the freezer chamber wall. Cotton swabs moistened with sterile 0.85% NaCl were used to collect samples from approximately 20 cm² areas during working hours. All samples were analyzed on the day of sampling at the Infectious Diseases and Tropical Medicine Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran.

Isolation of C. difficile
For culture analysis, a method of Rodriguez-Palacios et al. [3] was used. Briefly, 5 g of sample or swabs was added to 25 ml of selective enrichment C. difficile broth (1 liter containing 40 g proteose peptone, 5 g disodium hydrogen phosphate, 1 g potassium dihydrogen phosphate, 0.1 g magnesium sulfate, 2 g sodium chloride, 6 g fructose and 1 g sodium taurocholate supplemented with 500 mg cysteine hydrochloride, 12 mg norfloxacin and 32 mg moxalactam) in 50 ml falcon tubes and incubated for five to seven days at 37°C. For each sample, an alcohol shock to kill the vegetative cells was performed by adding a volume of 2 ml of enriched broth to an equal volume of absolute ethanol in a centrifuge tube, gently vortexed and kept at room temperature for 2 h. The samples were centrifuged at 10000 × g for 10 min, after which the supernatant was removed. The pellet was streaked onto Clostridium difficile Moxalactam Norfloxacin agar medium (CDMN agar) with 7% sheep blood and anaerobically incubated for 48 h at 37°C using an Anoxomat system (MART Microbiology B.V., Drachten, Netherlands). Isolates were presumptively identified as C. difficile by morphology, cresol/horse odor and L-proline β-naphthylamide disk (Prodisk, Hardy Diagnostics, Santa Maria, CA, USA). Culture and molecular assay were performed in a separate laboratory and negative controls were used in the entire process.

Molecular characterization of isolates
Suspected colonies were sub-cultured onto blood agar plates and incubated anaerobically at 37°C/24 h. DNA extraction was performed by transferring 3-5 colonies into 100 μl of sterile distilled water, heating at 95°C for 3 min and then centrifuged at 7500 × g for 15 min. The supernatant was used as a DNA-template PCR detection of genes encoding triose phosphate isomerase (tpi), toxin A and B (tcd A and tcd B, respectively) and binary toxin (cdtB) as described by Lemee et al. [18]; Stubbs et al. [19]. Isolates were also subjected to PCR-ribotyping as described by Bidet et al. [20]. Interpretation of ribotyping results was performed by visual identification. Ribotype patterns were designated (i.e. IR22) by internal nomenclature. A reference strain of ribotype 027 was available for comparison.

Results and discussion
C. difficile was isolated from 9/211 samples (4.2%; 95% CI [1.7]) from 2/7 (29%) processing plants (Table 1). These two processing plants (C and D) did not hold HACCP certification and were ranked in lowest level (category III) in terms of QAM and GMP. This is unsurprising because C. difficile contamination would be from carcass contamination by feces or intestinal contents, something that HACCP practices are designed to minimize. Similarly, lower rates of contamination of C. difficile in slaughterhouses have been reported when HACCP principles were implemented [11].

Raw beef meat was the only ingredient found to be contaminated with C. difficile in 3/54 (5.6%; 95% CI [1.15]). Detection of C. difficile in raw beef was unsurprising and demonstrates the potential of meat as a source of C. difficile dispersal, something that is logical given its

| Processing plant | Raw meat | Textured soy protein | Seasoning | Onion | Swab | Hamburger after molding | Hamburger after freezing | Total |
|------------------|----------|---------------------|-----------|-------|------|------------------------|------------------------|-------|
| A                | 0/8 (0)  | 0/2 (0)             | 0/3 (0)   | 0/2 (0) | 0/8 (0) | 0/4 (0) | 0/4 (0) | 0/31 (0) |
| B                | 0/6 (0)  | 0/2 (0)             | 0/3 (0)   | 0/2 (0) | 0/8 (0) | 0/4 (0) | 0/4 (0) | 0/29 (0) |
| C                | 1/8 (12.5%) | 0/2 (0)            | 0/1 (0)   | 0/2 (0) | 0/8 (0) | 0/4 (0) | 0/4 (0) | 1/29 (3.4) |
| D                | 2/8 (25%) | 0/2 (0)             | 0/4 (0)   | 0/2 (0) | 2/8 (25%) | 2/4 (50) | 2/4 (50) | 8/32 (25) |
| E                | 0/8 (0)  | 0/2 (0)             | 0/2 (0)   | 0/2 (0) | 0/8 (0) | 0/4 (0) | 0/4 (0) | 0/30 (0) |
| F                | 0/8 (0)  | 0/2 (0)             | 0/2 (0)   | 0/2 (0) | 0/8 (0) | 0/4 (0) | 0/4 (0) | 0/30 (0) |
| G                | 0/8 (0)  | 0/2 (0)             | 0/2 (0)   | 0/2 (0) | 0/8 (0) | 0/4 (0) | 0/4 (0) | 0/30 (0) |
| Total            | 3/54 (5.6%) | 0/14 (0)           | 0/17 (0)  | 0/14 (0) | 2/56 (3.5) | 2/28 (7.1) | 2/28 (7.1) | 9/211 (4.2) |
presence in food animals. However, *C. difficile* has been found in vegetables and other food items like fish, shellfish, edible bivalve molluscs, egg, ready to eat food (RTF) and salads [4,21-26], so while *C. difficile* is likely always of human or animal origin, it is important not to ignore the various potential sources that might have been contaminated by human or animal sources (e.g. manure contamination of water, indirect contamination by hands). If the main source of *C. difficile* contamination of hamburger is raw beef and this bacterium is endemic in the cattle population, it is likely that this organism can be introduced continuously.

The relatively low prevalence of *C. difficile* reported in the present study (5.6%) is in the range of recently published studies from numerous countries reporting the isolation of *C. difficile* from raw beef spanning 1.65 to 42.4% of samples collected at retail level [4,5,9,27,28]. Previous Iranian data are limited, with a report of contamination of 1.65% (2/121 samples) raw beef samples (9) and 2.8% (1/35) beef samples in meat packaging plants [29]. In contrast, this organism was not isolated from any of 145 raw chopped beef samples in the Netherlands [30].

Three different ribotypes were identified in raw beef (Table 2), with one accounting for 6/9 (67%) isolates. This strain was only found at one plant, but it was identified at two different timepoints. It is interesting to note that 1 out of 3 *C. difficile* strains was recovered from meat imported from Brazil. This strain had a distinct ribotype IR 21 pattern and was the only isolate obtained from processing plant C. The other contaminated beef samples originated from processing plant D. In both samples the meat was supplied from the same Iranian slaughterhouse but with different slaughtering dates. The commonness of one strain is not surprising if one (or a small number) of strains predominate in the Iranian cattle population, as is the case with ribotype 078 in many western countries. There is inadequate information about the *C. difficile* population structure in Iranian cattle to put these results into context. Another consideration would be cross contamination within the facility, resulting in numerous positive samples from one source. Laboratory contamination cannot be dismissed, but contamination of multiple samples from just one facility when samples from other facilities were being processed in parallel would be unlikely.

*C. difficile* was not found in any of the 45 non-meat ingredients of hamburger (14 textured soy proteins, 17 seasoning and 14 defrosted onions). Various potential explanations can be hypothesized. One is that the raw ingredients for these materials may be less likely to be contaminated. Another is that processing might have eliminated any *C. difficile* contamination, such as the heat generated during extrusion of textured soy protein [31].

The seasoning samples collected in this study were a mixture of black pepper, cinnamon, sumac and cumin were free of *C. difficile*. Seasonings could be contaminated with spores of *Clostridium* species because of the lack of proper sanitary conditions during collection or as a consequence of open air drying procedures [32]. However, in the processing plants selected in this study, the seasonings were supplied to the meat processing plants after being sterilized by irradiation in 2 KGy. A dose limit of irradiation for decontamination of microbial spores is in the range of 1-4 KGy [33], so the levels used here could have inactivated any *C. difficile* spores that might have been present.

Failure to isolate *C. difficile* from onions was not particularly surprising given the small sample size, but this bacterium has been isolated from onions in UK [34]. Similarly *C. difficile* was not found in a small number of onions in a Canadian study [22].

*C. difficile* spores are environmentally tolerant and resistant to many disinfectants, so it is not surprising to have found contamination in 2 out of 56 of environmental sites (3.5%; 95% CI [0, 12%]). The two strains that were found isolated from one processing plant (D) during two visits approximately 6 months apart, although it is unclear

### Table 2 Molecular characterization *C. difficile* strains isolated from hamburger and its ingredients in processing plants

| Source of isolation | Hamburger processing plant | Toxin gene profile | Ribotype | Sampling date |
|---------------------|----------------------------|--------------------|----------|---------------|
| Raw meat            | C                          | tcA tcB tcB cdtB   | IR21     | 17th November |
| Raw meat            | D                          | tcA tcB tcB cdtB   | IR22     | 4th September |
| Swab from grinder   | D                          | tcA tcB tcB cdtB   | IR22     | 4th September |
| Hamburger after molding | D                      | tcA tcB tcB cdtB   | IR22     | 4th September |
| Hamburger after freezing | D                      | tcA tcB tcB cdtB   | IR22     | 17th October  |
| Raw meat            | D                          | tcA tcB tcB cdtB   | IR23     | 17th October  |
| Swab from grinder   | D                          | tcA tcB tcB cdtB   | IR24     | 17th October  |
| Hamburger after molding | D                      | tcA tcB tcB cdtB   | IR22     | 26th November |
| Hamburger after freezing | D                     | tcA tcB tcB cdtB   | IR22     | 26th November |
whether this represents long-term persistence or (more likely) repeated contamination. Regardless, these results suggest that the hygiene procedures for cleaning and sanitation were not adequate. The standard operation procedure for sanitation used in processing plant D was washing the grinder with peracetic acid solution (4%) then rinsing with warm potable water. In the other plants where no C. difficile was found, the procedure was washing with sodium hydroxide solution (5%) followed by peracetic acid (4%) washing. The usage of oxidative agents and acids such as hypochlorite, hydrogen peroxide and peracetic acid is recommended to break the chemical bonds of food soils that build up the biofilm [35]. It has also pointed out that any residual organic material involved in biofilm formation could facilitate the attachment of spores and vegetative cells to meat processed in a grinder [11].

Of 28 hamburger samples taken at each step after molding and freezing, 4 (7.1%) were positive for C. difficile. As all positive isolates of C. difficile found in the same meat processing plant (D) belonged to an identical clone (RT 22), which was found on two separate occasions. This could suggest a common source of contamination, although inadequate data are available about strains found in food animals in Iran to properly interpret this finding. If this is a predominant food animal strain, the strain distribution noted here could simply reflect the background contamination of incoming meat products, although that would not explain the discrepancies in prevalence between facilities. Therefore, there must be concern that detection of the identical genotype (RT 22) from raw meat and final product at the same processing plant may indicate the persistence of this genotype during processing. In addition, colonization of the identical genotype in this processing plant may indicate the ineffectiveness of cleaning and sanitation. Complementary typing method such as multilocus variable-number tandem-repeat analysis (MLVA) or PFGE are needed to further investigate the persistence of C. difficile in the environment [36].

Detection of C. difficile from hamburger patties in 2/28 (7.1%; 95% CI [0, 23]) after 24 h freezing at −20°C indicates the survival of spores at freezing temperature, something that is well established in human fecal samples [14]. However there are no data on effect of freezing on survival of spores in meat.

The results of molecular characterizing of the 9 C. difficile strains are presented in Table 2. All isolates contained tcdB while 7 isolates from 2 strains also possessed tcdA. Interestingly, all isolates lacked cdtB. This was unexpected given the high prevalence of binary toxin positive strains reported in other studies of raw meat, the predominance of ribotype 078 (a cdt possessing strain) in food animals in various countries and the commonness of cdtB in human isolates from a recent study [6,9,13]. These results indicate that further study of the strain distribution of C. difficile in animals and humans in the region is required.

PCR ribotyping of 9 isolates categorized into four ribotypes (IR 21, IR22, IR23 and IR24) (Table 2). Ribotype IR 22 was the most frequently 6/9 (66.6%) encountered in our study. This genotype isolated from raw meat, environmental samples and hamburger both after forming and freezing in one processing plant (D), suggests that raw meat may be the major source of contamination. However the specific source of C. difficile in meat needs to be established. The gastrointestinal tract is the most important source of C. difficile contamination [9]. Transmission through animals’ hides, the slaughterhouse environment, the processing facility environment, processing equipment and the hands of personnel handling meat, must also be considered [9].

Inactivation of C. difficile spores by most of the cleaning and sanitation practices is difficult; therefore its accumulation in the environment increases the possibilities for contamination of meat. Ribotype IR 22 was isolated from various stages in processing plant D in both visits in the summer and autumn suggesting the excellent survival of spores in the processing plant. Ribotypes IR23 and IR24 were also found in processing plant D suggesting a relatively higher genetic diversity among C. difficile in this processing plant. Ribotype IR21 was the only distinct genotype found in beef meat imported from Brazil indicating the possibility of a geographical relationship of the genotypes.

Recently, hyper-virulent PCR ribotype 027 has emerged in North America and Europe in links to the hospital outbreaks [2]. This strain has not been reported yet in Iran in either food or clinical samples [9,17], potentially due to the lack of sufficient research in this area. Ribotype 027 also was not found in the present study, nor was ribotype 078, based on inference of the lack of cdtB in any isolate. We have previously reported ribotype 078 as a common strain in both humans and meat in Iran [9,17], so the absence of this strain in the current study was surprising.

This study is subjected to some limitations. First, sampling were conduced based on expiry date of ingredients where non-meat products kept for a significantly longer times. Therefore these products sampled less frequently than meat, resulting unbalanced sampling. Second limitation of this study is that only ribotyping used as strains characterization. Complementary typing methods such as MLVA or ideally whole genome sequencing would be needed to further investigate the epidemiology of C. difficile in the hamburger production facilities. Third, the possibility of lab cross-contamination of samples cannot be dismissed, although it may be unlikely for reasons cited above.

Conclusions
In conclusion, the study demonstrated the existence of toxigenic C. difficile in hamburger processing plants...
through different potential reservoirs such as raw meat and facilities. Occurrence of *C. difficile* in hamburger as a commonly consumed food in Iran is of public health significance although hyper-virulent strains of ribotype 027 and 078 were not found in this study. For reduction or prevention of *C. difficile* prevalence in food products, Good Manufacturing practices (GMP) and Hazard Analysis Critical Control Point (HACCP) system should be applied in food industries and followed by post-production control procedures by consumers such as proper cooking based on the adequate time for destruction of *C. difficile* spores [37]. Susceptible individuals with increased risk for development of CA-CDI should be also educated to minimize the exposure to this pathogen in the food supply [37].

**Abbreviations**

*C. difficile*: *Clostridium difficile*; CDI: *C. difficile* infection; CA-CDI: Community associated- *C. difficile* infection; PCR: Polymerase chain reaction.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

The project was performed by ZE, JSW, HE, MJ and MCh. PCR techniques were performed by ZE and MJ. Sample collection, culture, statistical analysis and manuscript writing were performed by ZE, JSW and MJ. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Akbar Ansarian in Deputy of Food and Drug in Isfahan University for his cooperation in statistical analysis. Statistical analysis and manuscript writing were performed by ZE, JSW and MJ. All authors read and approved the final manuscript.

**Author details**

1. Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran. 2. Department of Pathobiology and Centre for Public Health and Zoonoses, Ontario Veterinary College, University of Guelph, Guelph, Ont N1G2W1, Canada. 3. Department of Animal Science, Faculty of Agriculture and Natural Resources, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.

Received: 7 April 2014 Accepted: 4 November 2014

**Published online:** 25 November 2014

**References**

1. Martin H, Willey B, Low DE, Stampfli HR, McGee A, Boerlin P, Mulvey M, Weese JS. Characterization of *Clostridium difficile* strains isolated from patients in Ontario, Canada, from 2004–2006. *J Clin Microbiol* 2008, 46:2999–3004.

2. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC. Toxin Production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* 2005, 366:1079–1084.

3. Rodriguez-Palacios A, Stämpfli HR, Duffield T, Weese JS. *Clostridium difficile* in retail ground meat. *Emerg Infect Dis* 2007, 13:485–487.

4. Songer JG, Tinth HT, Killgore GE, Thompson AD, McDonald LC, Limbago BM. *Clostridium difficile* in meat products, USA. *J Clin Microbiol* 2007, 45:819–821.

5. Weese JS, Avey BP, Rousseau J, Reid-Smith RJ. Detection and enumeration of *Clostridium difficile* spores in retail beef and pork. *Appl Environ Microbiol* 2009, 75:5009–5011.

6. Curry SR, Marsh JW, Schlackman JL, Harrison LH. Prevalence of *Clostridium difficile* in uncooked ground meat products from Pittsburgh, Pennsylvania. *Appl Environ Microbiol* 2012, 78:4183–4186.

7. Suick EK, Putman M, Bermudez M, Thakur S. Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at the farm and slaughter. *Vet Microbiol* 2012, 157:172–178.

8. Quezada-Gomez C, Mulvey MR, Vargas P, Gamboa-Coronado NDM, Rodriguez C, Rodriguez-Cavallin E. Isolation of toxigenic and clinical genotype of *Clostridium difficile* in retail meats in Costa Rica. *J Food Prot* 2013, 76:348–351.

9. Rahimi E, Jalali M, Weese JS. Prevalence of *Clostridium difficile* in raw beef, cow, sheep, goat and camel and buffalo meat in Iran. *BMC Public Health* 2014, 14:119. doi:10.1186/1471-2458-14-119.

10. Hjung MA, Thompson AD, Killgore GE, Zukowski WE, Sanger G, Warny M, Johnson S, Gerdin DN, McDonald LC, Limbago BM. Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* 2008, 14:1039–1045.

11. Houser BA, Soehnlen MK, Wolfgang DR, Lysczek HR, Burns CM, Jayaro BM. Prevalence of *Clostridium difficile* toxin genes in the feces of veal calves and incidence of ground veal contamination. *Foodborne Pathog Dis* 2012, 9:32–36.

12. Harvey RB, Norman KN, Andrews K, Noy B, Hume ME, Scanlan CM, Hardin MD, Scott HW. *Clostridium difficile* in retail meat and processing plants in Texas. *J Vet Diagn Invest* 2011, 23:807–811.

13. Hawken P, Weese JS, Friesen M, Warriner K. Carriage and dissemination of *Clostridium difficile* and methicillin resistant *Staphylococcus aureus* in pork processing. *Food Control* 2013, 31:433–437.

14. Freeman J, Wilcox MH. The effects of storage conditions on viability of *Clostridium difficile* vegetative cells and spores and toxin activity in human faeces. *J Clin Pathol* 2003, 56:126–128.

15. Rodriguez-Palacios A, Reid-Smith RJ, Stämpfli HR, Weese JS. *Clostridium difficile* survives minimal temperature recommended for cooking ground meats. *Anz Veteriner* 2010, 16:540–542.

16. Rodriguez-Palacios A, Leisjue JT. Moist-heat resistance, spore aging, and uropoymorphy in *Clostridium difficile*. *Appl Environ Microbiol* 2011, 77:3085–3091.

17. Jalali M, Khorvash F, Warriner K, Weese JS. *Clostridium difficile* infection in an Iranian hospital. *BMJ Res Notes* 2012, 5:159. doi:10.1186/1756-0500-5-159.

18. Lerner IM, Dhallain A, Testelin S, Mattrat M, Maillard K, Lemeland J, Pons JL. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (toxin A), and tcdB (toxin B) genes for toxigenic culture of *Clostridium difficile*. *J Clin Microbiol* 2004, 42:5710–5714.

19. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerrden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* 2000, 186:307–312.

20. Bietd P, Barbut F, Lalinde V, Burghoffer B, Petit J. Development a new PCR ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett* 1999, 175:251–266.

21. Bakri MM, Brown DJ, Butcher JP, Sutherland AD. *Clostridium difficile* in ready-to-eat salads, Scotland. *Emerg Infect Dis* 2009, 15:817–818.

22. Metcalf DS, Costa MC, Dew WMW, Weese JS. *Clostridium difficile* in vegetables, Canada. *Lett Appl Microbiol* 2010, 51:600–602.

23. Pasquaile V, Romano VJ, Rupnik M, Dumontet S, Cimarron M, Aliberti F, Mauri F, Saggio M, Kroyvasek K. Identification and characterization of *Clostridium difficile* from shellfish and marine environments. *Folia microbiologica* 2011, 56:431–437.

24. Metcalf D, Avey BP, Janecko N, Matic N, Reid-Smith R, Weese JS. *Clostridium difficile* in seafood and fish. *Anzonaerob* 2011, 17:25–86.

25. Pasquaile V, Romano V, Rupnik M, Capuano F, Bove D, Aliberti F, Kroyvasek K, Dumontet S. Occurrence of toxigenic *Clostridium difficile* in edible bivalve molluscs. *Food microbiol* 2012, 31:309–312.

26. Obradovic V, Rupnik M. *Clostridium difficile* in meat products, eggs and vegetables in Slovenia. *The 4th International Clostridium difficile Symposium, European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Poster no. 116:8.*

27. Von Abecromie SM, Karlsson F, Wigh GT, Wierup M, Kroyvasek K. Low occurrence of *Clostridium difficile* in retail ground meat in Sweden. *J Food Prot* 2009, 72:1732–1734.

28. Rodriguez-Palacios A, Reid-Smith RJ, Stämpfli HR, Daignault D, Janecko N, Avey BP, Martin H, Thompson AD, McDonald LC, Limbago B, Weese JS. Possible seasonality of *Clostridium difficile* in retail meat, Canada. *Emerg Infect Dis* 2010, 15:802–805.

29. Esfandiar Z, Jalali M, Ezzatpanah H, Weese JS, Chamani M. Prevalence and characterization of *Clostridium difficile* in beef and mutton meats of Isfahan region, Iran. *Jundishapur J Microbiol* 2014, 7:e16771.
30. de Boer E, Zwartkruis-Nahuis A, Heuvelink AE, Harmanus C, Kuijper EJ: Prevalence of Clostridium difficile in retailed meat in the Netherlands. Int J Food Microbiol 2011, 144:561–564.
31. Guy R: Extrusion Cooking. Technologies and Applications. England: Woodhead Publishing Group Limited, CRC press, Cambridge; 2001:21.
32. Rahman MS: Handbook of Food Preservation. New York: Taylor & Francis Group, CRC Press; 2007:769.
33. Lara JD, Fernandez PS, Periago MP, Palop A: Irradiation of spores of Bacillus cereus and Bacillus subtilis with electron beam. Innovat Food Sci Emerg Technol 2002, 3:379–384.
34. Al Saif N, Brazier JS: The distribution of Clostridium difficile in the environment of South Wales. J Med Microbiol 1996, 45:133–137.
35. Cramer MC: Food Plant Sanitation: Design, Maintenance and Good Manufacturing Practices. New York: Taylor & Francis Group, CRC Press; 2006:19.
36. Wei HL, Kao CW, Wei SH, Tzen JTC, Chiou CS: Comparison of PCR ribotyping and multilocus variable-number tandem-repeat analysis (MLVA) for improved detection of Clostridium difficile. BMC Microbiol 2011, 11:217.
37. Rodríguez-Palacios A, Borgmann S, Kline TR, Lejeune JT: Clostridium difficile in foods and animals: history and measures to reduce exposure. Anim Health Res Rev 2013, 14:11–29.

doi:10.1186/s12866-014-0283-6
Cite this article as: Esfandiari et al.: Occurrence of Clostridium difficile in seasoned hamburgers and seven processing plants in Iran. BMC Microbiology 2014 14:283.