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ARTICLE

Prevalence of *Clostridium difficile* Isolated from Beef and Chicken Meat Products in Turkey

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Abstract The concern about the possibility of food can be a vehicle for the transmission of *Clostridium difficile* to humans has been raised recently due to the similarities among the strains isolated from patients, foods and food animals. In this study, therefore, the prevalence of *C. difficile* was investigated in beef and chicken meat products collected from 57 different butcher shops, markets and fast food restaurants in Sakarya province of Turkey. Two out of 101 samples (1.98%) was positive for *C. difficile* indicating a very low prevalence. The pathogen was isolated from an uncooked meatball sample and a cooked meat döner sample, whereas not detected in chicken meat samples. The meatball isolate was resistant to vancomycin and tetracycline, while the cooked meat döner isolate was resistant to vancomycin and metronidazole. Both isolates were sensitive to moxifloxacin and clindamycin. Toxins A and B were not detected. This study reveals the presence of *C. difficile* in further processed beef products in Turkey.

Keywords *Clostridium difficile*, meat, beef, chicken, antibiotic resistance

Introduction

Clostridium difficile is a gram-positive, obligate anaerobic, spore-forming and cytotoxin producing bacterium with optimum growth temperature at 35℃–40℃ (Dawson et al., 2009; Weese, 2010). The prevalence of *C. difficile* in healthy people's intestinal tract is 2%–3% and 40% in newborns (Libby and Bearman, 2009). It is recognized as a major cause of antimicrobial-associated and hospital-associated diarrhea, and the cause of almost all cases of pseudomembranous colitis (Weese, 2010). The two virulence factors associated with the *C. difficile* infection are toxin A (*tcd*A) and toxin B (*tcd*B), which are an enterotoxin and a cytotoxin, respectively (Kuehne et al., 2011). *C. difficile* was listed as one of the three urgent threats in the report on emerging pathogens with antibiotic resistance by the Centers for Disease Control and Prevention (Mooyottu et al., 2015). The gut microbiota changes, to be patient over 65 years old, previous hospitalization, long antibiotic therapy and underlying diseases are among the risk factors of *C. difficile* infection (Dawson et al., 2009; Gould and Limbago, 2010; Weese,

2010). Surviving of *C. difficile* spores on the surfaces for long periods of time and their resistance to many disinfectants are important factors that favor *C. difficile* spreading (Dawson et al., 2009).

It is considered that *C. difficile* can be transmitted to human by foods since it is disseminated by oral-fecal route and isolated from food animals including poultry. Meat can be contaminated by *C. difficile* through infected animals or food handlers during slaughtering (Rodriguez et al., 2013). Several studies determined that *C. difficile* spore contamination level is generally low (Bakri, 2018; Curry et al., 2012; Weese et al., 2009; Weese et al., 2010). However, the spores of this pathogen, if present in meat or other foods, may not be killed by cooking and can survive at 71℃ for two hours (Rodriguez-Palacios et al., 2010).

The high genotypic similarity among the strains isolated from patients, foods and food animals has increased the questions on the possibility that food can be a vehicle for transmission of *C. difficile* to humans (Rodriguez-Palacios et al., 2011; Rodriguez et al., 2013; Weese et al., 2011). The previous studies have shown that *C. difficile* prevalence is generally low in foods and the survey studies from year 2011 to 2018 have revealed that the prevalence of *C. difficile* in beef and chicken meat ranges from 1.6% to 12.4% (Bakri, 2018; De Boer et al., 2011; Esfandiari et al., 2014a, Esfandiari et al., 2014b, Esfandiari et al., 2015; Guran and Ilhak 2015; Kouassi et al., 2014; Quesada-Gómez et al., 2013; Rodriguez et al., 2014; Varshney et al., 2014; Visser et al., 2012). In a very recent report by Abdel-Glil et al. (2018), *C. difficile* was not cultured from poultry meat samples including retail chicken meat parts, chicken edible organs, duck meat parts and ducks' edible internal organs.

There is few data available on the prevalence of *C. difficile* in foods in Turkey (Guran and Ilhak, 2015). As far as we have known, this is the first report on the prevalence of *C. difficile* in cooked and further processed meat products in Turkey. Therefore, the aims of this study were as follows: (1) to determine the *C. difficile* prevalence in beef and chicken meat products, and (2) to characterize the toxigenic activities and antibiotic sensitivity patterns of *C. difficile* isolates.

Materials and Methods

Sample collection

Totally 101 samples of beef and chicken meat products (31 ground beef, 27 chicken breast, 18 meat ball, 12 cooked meat döner, 7 cooked chicken döner, 4 salami, 1 frankfurter and 1 bacon) were collected from 57 different butcher shops, markets and fast food restaurants from April 2013 to February 2014 in Sakarya province of Turkey. The collected samples, weighed not less than 100 g, were transferred to laboratory in an insulated icebox and analyzed in less than 24 h.

Isolation of *Clostridium difficile*

The isolation method used was based on the method described by Weese et al. (2009). Twenty-five g of each sample was placed into a sterile stomacher bag with filter (190×300 mm; LP Italiana, Milan, Italy) containing 25 mL of sterile phosphate-buffered peptone (PBS; 10 g/L peptone, 5 g/L NaCl, 9 g/L $Na_2HPO_4 \cdot 12H_2O$, 1.5 g/L K_2HPO_4, pH 7.0±0.2; Merck, Darmstadt, Germany) and homogenized by hand massaging for 5 min. From prepared homogenate, 1 mL portion was transferred into 9 mL of Clostridium Difficile Moxalactam Norfloxacin (CDMN; Oxoid, Hampshire, UK) broth added 0.1% sodium taurocholate and incubated at 37℃ for 48 h under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany). After enrichment procedure, alcohol shock was applied for spore selection. One mL of CDMN broth culture was mixed with 1 mL of anhydrous ethanol and the mixture was incubated at ambient temperature for 1 h. Following the incubation, the mixture was centrifuged at 4,000 rpm for 10 min. After supernatant was discarded, the pellet was streaked onto CDMN agar using sterile swab and the plates were incubated anaerobically at 37℃ for 48 h. The suspicious colonies grown on CDMN

agar were picked and transferred individually into Thioglycolate broth (Merck, Darmstadt, Germany) followed by anaerobic incubation at 37°C for 24 h.

Identification and confirmation

The suspicious colonies on CDMN agar were subcultured on Blood agar (Oxoid, Hampshire, UK). After anaerobic incubation at 37°C for 48 h, the plates were examined under UV light (365 nm). The isolates those were gram-positive, producing grey-white colonies with horse manure odor and yellowish-green fluorescent under UV illumination were subjected to L-proline aminopeptidase activity test (Pro-Disc, Remel Products, Lenexa, KS, USA). The L-proline aminopeptidase positive isolates were identified by API20A (Biomerieux, Marcy l'Etoile, France) and confirmed serologically by Clostridium Difficile Test Kit (DR1107A, Oxoid, Hampshire, UK).

Detection of toxin A/B

The *C. difficile* isolates were assayed for production of toxin A/B by using Xpect *C. difficile* Toxin A/B test (Thermo Fisher Scientific, Remel Products, Lenexa, KS, USA). The isolates were activated in Thioglycolate broth by incubating anaerobically at 37°C for 24 h. An appropriate amount of Thioglycolate broth culture was transferred to Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated anaerobically at 37°C for 72 h. The BHI broth culture was used to determine the toxin A/B according to the manufacturer instructions.

Antibiotic susceptibility testing

The susceptibilities of *C. difficile* isolates to metronidazole, vancomycin, moxifloxacin, tetracycline and clindamycin antibiotics were assayed using Epsilon test (E-test, Biomerieux, Marcy l'Etoile, France) on horse blood added Mueller-Hinton agar (Tenover et al., 2012). The plates were incubated at 37°C for 48 h. The minimum inhibition concentration (MIC) values for metronidazole, moxifloxacin, tetracycline and clindamycin were compared with the breakpoints established by Clinical and Laboratory Standards Institute (CLSI, 2018), while that for vancomycin with those defined by European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2018).

Results and Discussion

Totally 101 beef and chicken meat product samples collected from butcher shops, markets and restaurants were screened for *C. difficile* incidence. Totally 113 suspected isolates that yielded grey-white colonies on CDMN agar, yellow-green fluorescence under UV light with ρ-cresol odor were obtained. Fifty two isolates, which were positive with L-proline aminopeptidase test, were subjected to identification procedure using API20A. Two isolates were identified as *C. difficile* and confirmed serologically by Clostridium Difficile Test Kit (Table 1). Nineteen out of 52 isolates were identified as *Clostridium* species other than *C. difficile*. The most isolated species was *Clostridium beijenrickii/butyricum* and the others were *Clostridium ramosum*, *Clostridium botulinum/sporogenes, Clostridium septicum, Clostridium tertium, Clostridium bifermentas* and *Clostridium baratii*. This result shows that other *Clostridium* species display similar growth properties and colony morphology on CDMN agar as to *C. difficile*. Similarly, Limbago et al. (2012) isolated *Clostridium* species on CDMN agar, including *C. sporogenes*, *C. cadaveris*, *C. perfringens*, *C. bifermentas*, *C. septicum* and some other unidentified *Clostridium* spp.

Table 1. Results of identification with API20A

Identification result	Number of isolates
Clostridium difficile	2
Clostridium beijenrickii/butyricum	9
Clostridium ramosum	3
Clostridium botulinum/sporogenes	2
Clostridium septicum	2
Clostridium tertium	1
Clostridium bifermentas	1
Clostridium baratii	1
Non-*Clostridium*	13
Unidentified	18
Total	52

L-Proline aminopeptidase test is commonly used as a key test for detection of *C. difficile.* This test detects enzymatic hydrolysis of L-proline-β-naphthylamide based on releasing free β-napthylamine by red or pink color development after addition of ρ-dimethyl amino cinnamaldehyde reagent. Fedorko and Williams (1997) have suggested the ProDisc as a sensitive, specific and inexpensive method for confirmation of *C. difficile*. In current study, fifty two of 113 isolates gave positive results with L-proline aminopeptidase test; however, only two isolates were identified and confirmed as *C. difficile*. To develop more sensitive alternative methods for rapid detection of *C. difficile* in foods may be beneficial because the microbial flora of foods is different from fecal samples.

C. difficile was isolated from an uncooked meat ball sample and a cooked meat döner sample (Table 2), thus its incidence was 1.98% in tested samples. The previous studies have shown that the isolation rate of the pathogen is generally lower than 9% in beef and chicken meat (Bakri, 2018; De Boer et al., 2011; Esfandiari et al., 2014a, Esfandiari et al., 2014b, Esfandiari et al., 2015; Guran and Ilhak 2015; Jöbstl et al., 2010; Quesada-Gómez et al., 2013; Rodriguez et al., 2014; Varshney et al., 2014; Visser et al., 2012) and higher incidence levels, up to 42% were determined in several studies (Kouassi et al., 2014; Songer et al., 2009; Weese et al., 2009; Weese et al., 2010). In Europe relatively low prevalence rates up to 4.3% have been reported, whereas in North America the reported prevalence rates were higher up to 44% (Lund and Peck, 2015). One of the reasons for the different isolation rates may be the use of different methodologies for enrichment, isolation and identification of *C. difficile* as have been stated by Lund and Peck (2015). Although it is difficult to compare these incidence data due to the different isolation methods used, the current findings confirms the presence of *C. difficile* in meat products in Turkey.

The meat ball and meat döner samples, in which *C. difficile* was detected in current study (Table 3), are beef meat products prepared by hand that the contamination risk is high. On the other hand, the pathogen was not detected in the ground beef samples. Varshney et al. (2014) have mentioned that the prevalence of *C. difficile* in meat product may be largely affected by handling, processing and grinding. Because most cleaning and sanitation practices may most likely fail to inactivate *C. difficile* spores, it is possible that spores of this pathogen may accumulate on environment increasing contamination risk (Esfandiari et al., 2014b). In a study by Kalchayanand et al. (2013) *C. difficile* was not detected in 956 commercially produced ground beef samples. The authors have concluded that further processing of meat may increase the contamination risk of *C. difficile* spores from environment. Besides, they have mentioned the possibility of underestimation of *C. difficile* prevalence in ground

Table 2. Prevalence of *Clostridium difficile* in beef and chicken meat products

Sample type	Number of samples collected	Number of *C. difficile* positive samples
Ground beef	31	0
Chicken breast	27	0
Meat ball	18	1
Cooked meat döner	12	1
Cooked chicken döner	7	0
Salami	4	0
Frankfurter	1	0
Bacon	1	0
Total	101	2 (1.98%)

beef samples, since one gram of sample was used for enrichment that may reduce the isolation rate in case of low level *C. difficile* contamination.

C. difficile was isolated from one of the cooked meat samples. Döner is prepared by seasoning of meat with spices and the cone-like shaped meat mass is cooked by being slowly rotated in front of a heating element (Kayisoglu et al., 2003; Kilic, 2009). Because of its cooking method, the center of döner may be cold or warm when outside of the meat mass is cooked. Therefore, pathogenic microorganisms, including sporeformers may survive in cooked product (Kayisoglu et al., 2003). This result shows that spores of *C. difficile* may survive during cooking conditions of this product. Rodriguez-Palacios et al. (2010) reported that *C. difficile* spores survived for two hours at 71°C in pH-buffered solution. Additionally, it can survive at low-temperature conditions for up to four months (Deng et al., 2015). Songer et al. (2009) have reported that ready-to-eat products were more commonly *C. difficile* positive than were uncooked meats. On the other hand, Kouassi et al. (2014) have mentioned that heating may create anaerobic conditions depleting oxygen in cooked products and then if temperature is available the heat-activated spores may germinate and outgrow.

Some reports have mentioned that isolation rate of *C. difficile* is generally higher in winter than other seasons (Guran and Ilhak, 2015; Rodriguez-Palacios et al., 2009). In this study, the cooked meat döner and uncooked meat ball samples, from which *C. difficile* was detected, were provided in September and December months of the year 2013, respectively.

The two *C. difficile* isolates were tested using Epsilon test against five antibiotics including metronidazole, vancomycin, moxifloxacin, tetracycline and clindamycin. The MIC values are presented in Table 3. The meat döner isolate *C. difficile* ED046 was resistant to metronidazole and vancomycin, while the meat ball isolate *C. difficile* MB025 was resistant to vancomycin and tetracycline based on the breakpoints defined by CLSI and EUCAST. Metronidazole and vancomycin are the antibiotics recommended by European Society of Clinical Microbiology and Infectious Diseases (ESCMID) for treatment of non-severe and severe *C. difficile* infections, respectively (Bauer et al., 2009). Also, clindamycin and moxifloxacin are among the antibiotics used for *C. difficile* infection treatment (Varshney et al., 2014). According to Huang et al. (2009) the resistance to metronidazole and vancomycin is very rare, however decreased sensitivity is emerging. Varshney et al. (2014) determined 100% susceptibility to metronidazole and vancomycin among the *C. difficile* strains isolated from meat samples. Wong et al. (1999) determined only one out of one hundred *C. difficile* isolates was resistant to metronidazole. The resistance rates of 953 *C. difficile* isolates to metronidazole and vancomycin were reported by Freeman et al. (2015) as 0.11 and 0.87%, respectively.

The toxins A and B were not detected in BHI broth cultures of two *C. difficile* isolates. According to the recent reports,

Table 3. MIC values of antibiotics for *Clostridium difficile* isolates by E-test

Antibiotics	MIC breakpoints (μg/mL)			MIC values of *C. difficile* isolates (μg/mL)	
	S	I	R	MB025	ED046
Metronidazole[1)]	≤8	16	≥256	0.125	>256
Moxifloxacin[1)]	≤2	4	≥8	0.75	0.38
Tetracycline[1)]	≤4	8	≥16	48	0.047
Clindamycin[1)]	≤2	4	≥8	0.6	0.19
Vancomycin[2)]	≤2	-	>2	>256	4

[1)] The breakpoints defined by Clinical and Laboratory Standards Institute (CLSI).
[2)] The breakpoints defined by European Committee for Antimicrobial Susceptibility Testing (EUCAST).
MIC, minimum inhibition concentration; S, susceptible; I, intermediate; R, resistant.

toxigenic *C. difficile* may be present in meat products, usually at a low concentration (Esfandiari et al., 2014a; Guran and Ilhak, 2015). The existence of non-toxigenic but antibiotic resistant *C. difficile* strains in foods may be considered as a potential public health risk. Mooyottu et al. (2015) detected *C. difficile*, non-toxigenic but with antibiotic resistance genes, in two out of 300 meat samples including beef, chicken and pork meat and they stated that there is a risk for formation of toxigenic *C. difficile* by horizontal gene transfer.

A better understanding of *C. difficile* contamination of food products is required to assess the role of foods in *C. difficile* infections; hence, different types of meat products including cooked or further processed meats were analyzed for the presence *of C. difficile*. The results of the current study confirm the existence of *C. difficile* in beef products subjected to further processing such as grounding, seasoning, cooking etc. The isolated *C. difficile* strains were not toxigenic. However, their resistance to vancomycin and metronidazole, which are two main antibiotics used for treatment of *C. difficile* infection, is noteworthy in respect to a possible gene transfer between toxigenic and non-toxigenic strains.

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