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ARTICLE

Raw Animal Meats as Potential Sources of *Clostridium difficile* in Al-Jouf, Saudi Arabia

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Abstract *Clostridium difficile* present in feces of food animals may contaminate their meats and act as a potential source of *C. difficile* infection (CDI) to humans. *C. difficile* resistance to antibiotics, its production of toxins and spores play major roles in the pathogenesis of CDI. This is the first study to evaluate *C. difficile* prevalence in retail raw animal meats, its antibiotics susceptibilities and toxigenic activities in Al-Jouf, Saudi Arabia. Totally, 240 meat samples were tested. *C. difficile* was identified by standard microbiological and biochemical methods. Vitek-2 compact system confirmed *C. difficile* isolates were 15/240 (6.3%). Toxins A/B were not detected by Xpect *C. difficile* toxin A/B tests. Although all isolates were susceptible to vancomycin and metronidazole, variable degrees of reduced susceptibilities to moxifloxacin, clindamycin or tetracycline antibiotics were detected by Epsilon tests. *C. difficile* strains with reduced susceptibility to antibiotics should be investigated. Variability between the worldwide reported *C. difficile* contamination levels could be due to absence of a gold standard procedure for its isolation. Establishment of a unified testing algorithm for *C. difficile* detection in food products is definitely essential to evaluate the inter-regional variation in its prevalence on national and international levels. Proper use of antimicrobials during animal husbandry is crucial to control the selective drug pressure on *C. difficile* strains associated with food animals. Investigating the protective or pathogenic potential of non-toxigenic *C. difficile* strains and the possibility of gene transfer from certain toxigenic/ antibiotics-resistant to non-toxigenic/antibiotics-sensitive strains, respectively, should be worthy of attention.

Keywords animal meat, diarrhea, pseudomembranous colitis, resistance, spores

Introduction

Clostridium difficile is a dangerous organism that is responsible for 15%–30% of antibiotic associated diarrhea cases around the world (Hampikyan et al., 2018). Many important risk factors such as improper use of antibiotics, reduced immunity and advanced age of the host may facilitate acquiring of *C. difficile* infection (CDI) (Rupnik et al., 2009). Centers for Disease Control and Prevention listed *C. difficile* between the most dangerous three urgent emerging multi-antibiotics resistant pathogens

(Mooyottu et al., 2015). The infected persons may suffer from mild diarrhea, pseudo-membranous colitis, toxic megacolon or even death (ECDC, 2018).

Lawson et al. (2016) reclassified *C. difficile* as *Clostridioides difficile* which is an anaerobic, Gram-positive, spore-forming bacterium. It grows best at 35°C–40°C (Dawson et al., 2009). Surviving of *C. difficile* spores on the surfaces for long times and their resistance to many disinfectants are important factors that favor spreading of the organism (Weese et al., 2010). The spores, if contaminated the meat from food handlers during slaughtering or from the infected animals, may survive for two hours at 71°C, so they are not be killed by cooking (Rodriguez et al., 2013).

There is change in *C. difficile* epidemiology with increasing incidence, severity, relapses of CDI in humans after the emergence of the novel hypervirulent strains, as 078 and 027 ribotypes, in North America and Europe (Smits et al., 2016). Young non-hospitalized persons, who were earlier considered as a low-risk group, now can be affected by CDI. Furthermore, in Netherlands and USA there are remarkable rates of probable community-acquired CDI (Abdel-Glil et al., 2018).

The human carrier rates of *C. difficile* vary from high percent (15%) in Japan to low percent (0%–3%) in Europe (Mulligan, 2008). Similarly, animals can act as carriers for *C. difficile* (Keessen et al., 2011). Therefore, *C. difficile* can contaminate soil, foods and water through feces, and this could suggest a possible method of transmission to humans resulting in CDI (Abdel-Glil et al., 2018).

If livestock are potential sources of *C. difficile*, food products contaminated with their feces could be one of the transmission modes from infected or colonized animals to humans through the food chain. It was reported that shedding of *C. difficile* during slaughtering of animals and spillage of their gut contents during evisceration can result in accumulation of *C. difficile* spores within the slaughterhouse environment leading to contamination of the animal carcasses and meats (EFSA, 2013).

CDI has a major cost impact with an estimated annual cost of U.S. $3.2 billion (Zilberberg et al., 2008). The prevalence rate of CDI among patients with diarrhea in Egypt is 23.6% (Abu Faddan et al., 2016), in Lebanon is 65.2% (Moukhaiber et al., 2015) and in Jordan is 92.4% (Wadi et al., 2015). In Saudi Arabia, there is no published study about prevalence of CDI on a national level, yet, few reports of single-center studies detected low rate of CDIs (Obaid and Alhifany, 2020). One of these studies reported 4.6% prevalence rate of CDI among patients with diarrhea (Shehabi et al., 2015). Another study reported an increase in the prevalence rate of healthcare-associated CDIs from 17% in 2001 to 20% in 2018 among all suspected diarrheal stool tested (Al-Tawfiq et al., 2020).

Data about *C. difficile* susceptibility to antibiotics are important for better estimating the organism's virulence and predicting its management plan (Peng et al., 2017). *C. difficile* resistance to antibiotics and its production of toxins play major roles in the pathogenesis of CDI (Kuehne et al., 2011). Vancomycin and metronidazole were recommended as a treatment of CDI (Debast et al., 2014; Cho et al., 2020). Moreover, clindamycin, tetracycline and moxifloxacin are among the most significant risk antibiotics for developing of CDI (Teng et al., 2019). Recently, concerns about the prophylactic and therapeutic use of many antibiotics, such as vancomycin, metronidazole and fluoroquinolones, in butchery animal husbandry to promote their growth have gradually increased (Muratoglu et al., 2020).

Toxins are the most important virulence factors responsible for CDI in addition to other factors (Janoir, 2016). Toxin A is an enterotoxin that can lead to accumulation of fluids in colon of many animal models. Toxin B is a cytotoxin that can lead to inflammation and damage of mucosa of the colon (Voth and Ballard, 2005). These two toxins with their regulatory genes are chromosomally encoded in a specific pathogenicity locus (*PaLoc*) that is absent in the non-toxigenic strains (Martin-Verstraete et al., 2016). It should be noted that approximately 11% of the *C. difficile* genome is made up of mobile genetic elements that could facilitate modulation of toxin gene expression, the transfer of antibiotic resistance or toxin genes and the

conversion of toxin non-producers into toxigenic strains (Mooyottu et al., 2015; Peng et al., 2017).

A better understanding of *C. difficile* transmission from animals to humans is required all over the world. Information on *C. difficile* isolation and characterization from many animal meat products has amplified quickly in different countries and populations; however, such information is not sufficient in Saudi Arabia. As far as I know, this is the first study to determine the prevalence of *C. difficile* in raw camel, cow, sheep, and goat meats that were collected from Sakaka, Al-Jouf, Saudi Arabia and to evaluate the isolates' antibiotics sensitivity patterns and toxigenic activities.

Materials and Methods

Collection of samples

Bioethical approval was obtained from the local committee of bioethics (LCBE) of Jouf University, Saudi Arabia (approval No: 07-02/41). A cross-sectional study was conducted to collect 240 raw animal meat samples (60 from camels, 60 from cows, 60 from sheep, and 60 from goats) in October and November of the year 2019. The samples were randomly purchased (by simple random sampling procedure; flipping a coin) from 25 retail outlets (butcher shops, markets and supermarkets) in Sakaka, Al-Jouf, Saudi Arabia. Each sample, at least 100 g weight, was collected in a sterile bag, and transported in an icebox to microbiology laboratory for processing.

Isolation and identification of *C. difficile*

The samples were processed using aseptic techniques to avoid their contamination as described by Weese and colleagues (Weese et al., 2009). Briefly, 25 g from each sample was homogenized by hand massaging for 5 min with 25 mL of sterile phosphate buffered peptone (PBP) inside a sterile bag. From the prepared homogenate, 1 mL was mixed with 9 mL of *C. difficile* Moxalactam Norfloxacin (CDMN) broth (Oxoid, Hampshire, UK) with 0.1% sodium taurocholate then incubated at 37℃ anaerobically for 7 days by using anaerobic jars with gas packs and anaerobic indicators (Oxoid). Selection of spores was done by alcohol shock as the following; 1 mL of CDMN broth culture was mixed with equal volume of anhydrous ethanol, incubated for 1 h at ambient temperature, centrifuged for 10 min at 1,792×g, the supernatant was discarded then the pellet was inoculated on CDMN agar by using a sterile swab then incubated at 37℃ anaerobically for 72 h. Suspicious growth on the CDMN agar was subcultured into thioglycolate broth then incubation at 37℃ under anaerobic conditions for 72 h. Likewise, suspicious growth on the CDMN agar was subcultured on blood agar. After incubation under anaerobic conditions at 37℃ for 72 h, suspected colonies were examined by the standard microbiological and biochemical techniques including colony morphology and odor testing and Gram staining.

Confirmation of *C. difficile*

Suspected colonies (greyish white with horse manure odor and revealing Gram-positive bacilli) were examined by L-proline aminopeptidase and *C. difficile* test kits (Oxoid) as per the manufacturer's instructions. The positive isolates were confirmed by Vitek-2 compact system (BioMérieux, Marcy l'Etoile, France). A control positive reference strain (ATCC 9689) was included in all steps (Oxoid) (ECDC, 2018).

Toxins A/B detection

Toxins A/B production by the confirmed *C. difficile* isolates was evaluated by Xpect CD Toxin A/B test (Oxoid) according

to the supplier's manual. Triplicate testing was done for each isolate. Briefly, thioglycolate broth of isolates was incubated at 37℃ anaerobically for 24 h. Sufficient volume of the broth culture was mixed with an equal volume of brain heart infusion (BHI) broth and incubated anaerobically at 37℃ for 72 h then used to detect the toxins (ECDC, 2018). *C. difficile* ATCC 9689 (Oxoid) was used as a positive control strain (toxigenic $A^+/B^+/CDT^-$).

Antibiotic susceptibility testing

The Vitek-2-confirmed *C. difficile* isolates susceptibility/resistance to vancomycin, metronidazole, tetracycline, clindamycin and moxifloxacin antibiotics was evaluated by Epsilon tests (E-tests, BioMérieux) according to the manufacturer's manual. *C. difficile* ATCC 9689 (Oxoid) was used as a positive control reference strain. Triplicate testing was performed for each isolate. The isolates were inoculated on brucella agar (Oxoid) supplemented with 5.0% sheep blood. Two minimum inhibition concentration (MIC) evaluator strips were placed on the agar then the plates were incubated at 37℃ anaerobically for 72 h. Vancomycin MIC values were compared with the European committee for antimicrobial susceptibility testing (EUCAST, 2019) breakpoints, while MIC values of metronidazole, tetracycline, clindamycin and moxifloxacin were compared with the clinical and laboratory standards institute (CLSI) breakpoints (CLSI, 2019).

Data analysis

C. difficile prevalence was compared between animal meat types by Chi-square and Fisher exact tests. Statistical significance was considered at $p<0.05$.

Results

Contamination of raw animal meats by *C. difficile* was screened in 240 meat samples. One hundred isolates were suspected (greyish white, rounded with a distinctive horse manure odor on CDMN agar). Fifty-five of them were positive by L-proline aminopeptidase and *C. difficile* test kits. *C. difficile* was confirmed by Vitek-2 compact system from 15/240 (6.3%) raw animal meat samples. Furthermore, Other *Clostridium* species were identified (Table 1). A statistical significance ($p=0.019$) was detected in *C. difficile* prevalence between different animal meat samples (Table 2). It was clear that contamination of cow meats is more prevalent followed by camel meats.

Although all Vitek-2 compact system-confirmed *C. difficile* isolates were susceptible to vancomycin and metronidazole antibiotics, some isolates were intermediate/resistant to tetracycline, clindamycin or moxifloxacin with variable degrees (Table 3). Toxins (A and B) were not detected among all confirmed *C. difficile* isolates.

Discussion

Food contamination with feces of colonized or infected livestock animals could be one of the transmission routes of *C. difficile* from animals to humans via the food chain. *C. difficile* has been detected in a wide range food, from beef (Rodriguez et al., 2014), pork (Rodriguez et al., 2016), chicken meats (de Boer et al., 2011; Taha, 2021) to raw milk (Romano et al., 2018), vegetables (Eckert et al., 2013) and seafood (Troiano et al., 2015), taken directly from the grocery stores worldwide. The presence of *C. difficile* spores in these end products can be explained by initial contamination of their raw materials, cross-contamination during their industry or production of the spores during their processing (Gauvry et al., 2017). In the domestic environment, spores present in refrigerators and on kitchen surfaces can contaminate the food products (Weese et

Table 1. Results of isolates identification by Vitek-2 compact system

Identification result	Number of isolates
Clostridium difficile	15
Clostridium bifermentans	4
Clostridium sordellii	4
Clostridium tertium	2
Clostridium baratii	1
Clostridium glycollicum	1
Clostridium ramosum	1
Clostridium septicum	1
Non-*Clostridium*	8
Unidentified	18
Total	55

The sample size was calculated on line (https://www.surveysystem.com/sscalc.htm#one) with confidence interval 6.32 at 95% confidence level and 250,000 Sakaka populations.

Table 2. Prevalence of *Clostridium difficile* in different animal meat samples

Sample type	Number of samples collected	*C. difficile* positive samples: Number (%)
Cow meat	60	8 (13.3)*
Camel meat	60	5 (8.3)
Sheep meat	60	1 (1.7)
Goat meat	60	1 (1.7)
Total	240	15 (6.3)

* The chi-square statistic is 9.88. The p-value is 0.019. The result is significant at $p \leq 0.05$. It was clear that contamination of cow meats is more prevalent followed by camel meats.

al., 2010).

Variable methods and culturing techniques can be used for *C. difficile* detection in food products due to absence of a gold standard procedure. The variability in the methodologies preclude the data comparison from different studies (Rupnik and Songer, 2010). In the current study, only 15 *C. difficile* isolates were confirmed by the Vitek-2 compact system among 240 tested raw animal meat samples. In addition, Other *Clostridium* species (most of them were *C. bifermentans* and *C. sordellii*) that displayed similar growth characters and colony morphology on CDMN agar were detected (Table 1). Similarly, Limbago et al. (2012) reported many *Clostridia* with similar growth characters on CDMN agar, as *C. cadaveris*, *C. sporogenes*, *C. bifermentas*, *C. perfringens*, *C. septicum*, *C. difficile* and some other unidentified *Clostridia*. These *Clostridia* may cross-react with *C. difficile* during its identification by L-proline aminopeptidase and *C. difficile* test kits. Consequently, in the conducted study, confirmation was done by Vitek-2 compact system with including a particular positive control reference strain of *C. difficile* (ATCC 9689) in each experiment. Other studies used Api 20A (Kouassi et al., 2014), API Rapid ID 32A (Troiano et al., 2015) or molecular (Bakri, 2018; Romano et al., 2018; Zhang et al., 2019; Usui et al., 2020) tests to confirm *C. difficile* isolates.

In the conducted study, the detected contamination level of raw animal meats by *C. difficile* was low (6.3%). Many previous studies from different countries reported a contamination level of animal meats by *C. difficile* lower than 9% (Bakri,

Table 3. Minimum inhibitory concentration (MIC) values of selected antibiotics against *Clostridium difficile* isolates by E-tests

Anti-biotics	MIC (μg/mL) breakpoints			Number of *C. difficile* isolates (%)			MIC values (μg/mL) of *C. difficile* isolates and control														
	S	I	R	S (%)	I (%)	R (%)	Iso-late (1)	Iso-late (2)	Iso-late (3)	Iso-late (4)	Iso-late (5)	Iso-late (6)	Iso-late (7)	Iso-late (8)	Iso-late (9)	Iso-late (10)	Iso-late (11)	Iso-late (12)	Iso-late (13)	Iso-late (14)	Iso-late (15)	ATCC 9689
Vanco-mycin[1)]	≤2	-	>2	15 (100)	0 (0)	0 (0)	0.5	1.0	0.5	0.5	0.25	0.25	1.0	0.25	2.0	0.5	1.0	1.0	0.5	0.25	0.25	0.5
Metroni-dazole[2)]	≤8	16	≥32	15 (100)	0 (0)	0 (0)	0.5	0.5	0.25	1.0	0.03	8.0	0.5	1.0	0.5	0.5	0.06	4.0	8.0	0.25	1.0	2.0
Tetracy-cline[2)]	≤4	8	≥16	10 (66.7)	5 (33.3)	0 (0)	8.0	0.25	0.015	0.25	4.0	8.0	2.0	0.03	8.0	0.03	8.0	0.015	0.06	8.0	4.0	4.0
Clinda-mycin[2)]	≤2	4	≥8	11 (73.3)	4 (26.7)	0 (0)	0.5	4.0	4.0	2.0	4.0	0.5	1.0	4.0	2.0	1.0	0.25	0.25	0.25	1.0	0.25	1.0
Moxi-floxacin[2)]	≤2	4	≥8	6 (40.0)	6 (40.0)	3 (20.0)	0.5	4.0	8.0	4.0	8.0	0.25	4.0	0.25	0.25	4.0	1.0	4.0	8.0	4.0	0.25	2.0

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

2018; de Boer et al., 2011; Esfandiari et al., 2014a, Esfandiari et al., 2014b, Esfandiari et al., 2015; Jöbstl et al., 2010; Lund and Peck, 2015; Quesada-Gómez et al., 2013; Rodriguez et al., 2014; Varshney et al., 2014). Contrary to these results, Bouttier et al. (2010) in France and Pires et al. (2018) in Brazil, reported that they did not detect any *C. difficile* isolate from 59 and 80 animal meat samples, respectively. On the other hand, higher detection rates, up to 42% were reported by some studies (Kouassi et al., 2014; Weese et al., 2009). Lund and Peck (2015) have reported a higher rate (44%) in North America.

Among the reasons for variability in *C. difficile* detection rates may be the variability in the methodologies used for enrichment, isolation, identification and confirmation of the isolates (Lund and Peck, 2015). Another reason may be the variability in the degree of meat samples processing. Songer et al. (2009) have reported that uncooked meats were less commonly contaminated by *C. difficile* than ready-to-eat meat products. Many studies have reported the increase in *C. difficile* detection rates with more handling, grinding and processing due to failure of most cleaning and sanitation practices to inactivate the spores that may accumulate on more environmental surfaces with increasing the possibility of meat contamination (Esfandiari et al., 2014b; Varshney et al., 2014).

It was clear in the current study that contamination of cow meats is more prevalent followed by camel meats. This might be due to more contact of humans with cows and camels on a daily basis to get their milk. Furthermore, farmers keep cows most of the time in cowsheds that are usually close to their houses and this increases the possibility of *C. difficile* transmission between humans and cows.

Resistance of *C. difficile* to antibiotics plays an important role in development of CDI. The most commonly reported risk factor for development of CDI in humans is the prolonged use of antibiotics that could disrupt the colonic microbiota resulting in *C. difficile* overgrowth (Kuehne et al., 2011). Fifteen confirmed *C. difficile* isolates were tested against five antibiotics including vancomycin, metronidazole, tetracycline, clindamycin and moxifloxacin. Tetracycline, clindamycin and moxifloxacin are major risk antibiotics for CDI development (Teng et al., 2019). Vancomycin and metronidazole were recommended for treatment of severe and non-severe CDIs, respectively (Debast et al., 2014). Recently, it was reported that the use of metronidazole alone for treatment of non-severe CDIs is associated with higher recurrence rates. Consequently, metronidazole was recommended for treatment of non-severe CDIs only if vancomycin and fidaxomicin are not tolerated or

unavailable. Fulminant cases need combination of vancomycin with metronidazole (Cho et al., 2020).

Although all isolates in the conducted study were susceptible to vancomycin and metronidazole antibiotics, variable degrees of reduced susceptibility to tetracycline, clindamycin or moxifloxacin were detected in some isolates (Table 3). This result is in agreement with Varshney et al. (2014) and Berger et al. (2020) who reported complete susceptibility of *C. difficile* strains isolated from meat samples to vancomycin and metronidazole. Furthermore, Freeman et al. (2015) reported the resistance to vancomycin and metronidazole among 953 *C. difficile* isolates as 0.87% and 0.11%, respectively. Moreover, Muratoglu et al. (2020) and Taha (2021) detected only one out of 22 and 11 *C. difficile* isolates was resistant to metronidazole, respectively. On the other hand, Ersöz and Coşansu (2018) detected one tetracycline-vancomycin resistant *C. difficile* isolate recovered from uncooked meatball and another metronidazole-vancomycin resistant *C. difficile* isolate recovered from cooked meat sample.

The current study detected 4/15 clindamycin-intermediate, 6/15 moxifloxacin-intermediate and 3/15 moxifloxacin-resistant *C. difficile* isolates. Berger et al. (2020) reported 2/80 clindamycin-resistant and 26/80 moxifloxacin-resistant isolates. The relative decrease in the susceptibility of *C. difficile* to moxifloxacin might be cross-resistance with other fluoroquinolones which might be used for treatment of multiple gastrointestinal infections.

The variability of reported results regarding antibiotic susceptibility of *C. difficile* isolates from animal meat origins can be explained by exposure of the food animals to different antibiotics during farm rearing or differences in the genetic characters of the strains.

The toxins A and B were not detected in the broth cultures of the 15 confirmed *C. difficile* isolates. This result is consistent with the results of two studies in which 100.00% of *C. difficile* isolates detected in animal meats were non-toxigenic (Ersöz and Coşansu, 2018; Mooyottu et al., 2015). Furthermore, some studies reported predominance of the non-toxigenic *C. difficile* isolates at rates 66.70% and 76.30% (Jöbstl et al., 2010; Wu et al., 2017), respectively. In contrast, some researchers reported that majority of the *C. difficile* isolates were toxigenic at rates 78.50%, and 88.80% (Bakri, 2018; Rodriguez et al., 2014), respectively. In addition, some reports detected 100.00% toxigenic *C. difficile* isolates (Bouttier et al., 2010; Esfandiari et al., 2014a; Esfandiari et al., 2014b; Muratoglu et al., 2020).

Some reports considered the existence of non-toxigenic *C. difficile* strains in meat products could be a potential public health problem by generation of toxigenic strains through horizontal gene transfer (Mooyottu et al., 2015; Peng et al., 2017). On the other hand, other reports considered non-toxigenic *C. difficile* strains isolated from samples of human, environmental or animal origin, including food products, are non-pathogenic. Furthermore, some reports proved a protective role of colonization by these non-toxigenic strains against the toxigenic ones in the hamster model (Janoir, 2016).

More studies in animal models and humans are needed to evaluate the protective or pathogenic potential of non-toxigenic *C. difficile* strains and to examine the possibility acquiring the *PaLoc* genes by toxin-negative strains to express clinically relevant levels of toxins.

Conclusion

A better understanding of *C. difficile* contamination of animal meats is required to assess their role in CDIs all over the world. As far as I know, the conducted study is the first one in Al-Jouf region, Saudi Arabia, to evaluate this possibility. The study detected a low contamination level by non-toxigenic strains with different degrees of reduced susceptibility to some antibiotics. Variability between the worldwide reported *C. difficile* contamination levels could be due to absence of a gold

standard procedure for its isolation. The establishment of a unified screening and testing algorithm for *C. difficile* detection in food products is definitely essential to evaluate the inter-regional variation in its prevalence on national and international levels. It is highly recommended to include and compare *C. difficile* susceptibility/resistance data in future studies and combine these data with nucleic acid amplification testing for better understanding of its virulence and suspecting its best empirical treatment. Proper use of antimicrobials during butchery animal husbandry is crucial to control the selective drug pressure on *C. difficile* strains associated with food animals. Investigating the protective or pathogenic potential of non-toxigenic *C. difficile* strains and the possibility of gene transfer from certain toxigenic and antibiotics-resistant strains to non-toxigenic and antibiotics-sensitive strains, respectively, should be worthy of attention to avoid CDI especially for persons who are immune-compromised or on broad spectrum antibiotics for long periods.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

The article is prepared by a single author.

Ethics Approval

Approval was obtained from the local committee of bioethics (LCBE) of Jouf University, Saudi Arabia (approval No: 07-02/41).

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