Original Article

Retail chicken meats as potential sources of *Clostridioides difficile* in Al-Jouf, Saudi Arabia

Ahmed Elsayed Taha Attia[1,2]

[1] Microbiology and Immunology unit, Department of Pathology, College of Medicine, Jouf University, Sakaka City, Al-Jouf, Saudi Arabia
[2] Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura City, Egypt

Abstract
Introduction: Presence of *Clostridioides difficile* in stool of food birds and animals is a risk for contamination of their meats to become potential sources of human infection. The main virulence factors of *C. difficile* are its resistance to antibiotics, production of toxins and spores. As far as I know, this is the first study to evaluate *C. difficile* prevalence in chicken meats, its toxigenic activities and antibiotics sensitivity patterns in Al-Jouf, Saudi Arabia.
Methodology: Totally, 250 raw chicken meat samples were examined. Standard microbiological and biochemical procedures were used for *C. difficile* isolation and identification. The suspected colonies were tested by L-proline and *C. difficile* test kits then confirmed by Vitek 2 compact system. Xpect *C. difficile* toxin A/B test was used to detect A/B toxins production. Antibiotics susceptibility patterns were detected by Epsilon tests.
Results: *C. difficile* was isolated from 11/250 (4.40%) chicken meat samples; 5/65 (7.69%) legs, 3/65 (4.61%) thighs, 2/60 (3.33%) wings and 1/60 (1.67%) breasts ($p = 0.4$). All isolates were non-toxigenic. Although all isolates were vancomycin sensitive, some isolates were intermediate/resistant to metronidazole, tetracycline, clindamycin or moxifloxacin antibiotics with variable degrees.
Conclusions: *C. difficile* might contaminate retail chicken meats. Although low level of contamination by non-toxigenic strains was detected, chicken meats should be investigated as *C. difficile* infection sources for humans especially elders, immune-compromised and long terms wide spectrum antibiotics-used persons. Decreased sensitivity of *C. difficile* to antibiotics is emerging.

Key words: antibiotics; contamination; resistance; spores; toxins; Saudi Arabia.

Introduction

Clostridioides difficile (*C. difficile*; formerly *Clostridium difficile*) is an enteropathogen in humans, some animals and birds [1]. The resistant spores of this anaerobic Gram-positive bacterium can contaminate foods since they resist low temperature for up to four months [2], heating for up to two hours at 71 °C and chemicals. Furthermore, spores can resist cooking conditions and stomach acidity to cause *C. difficile* infection (CDI) [3].

Increasing concern towards this organism has been started because of the international increase in morbidities, mortalities and relapses that are largely associated with emergence and dissemination of hypervirulent strains, as 078 and 027 ribotypes [4]. Furthermore, an international increase in recognition of community-associated *C. difficile* infection (CA-CDI)

has been noted in people that were considered at low risk of this fatal disease [5].

Globally, over the previous two decades, the rates of CDI have considerably increased. Although CDI may be asymptomatic, it may present with diarrhea, pseudomembranous colitis, toxic megacolon or even death. The number of cases and deaths associating CDI is increasing in both healthcare settings (especially if elderly with a history of long-term antibiotic therapy that disrupts the normal intestinal microbiota) and communities (without a prior history of antibiotic therapy or hospitalization) [6-9]. In Saudi Arabia, reports are inadequate about CDIs' methods of screening, mortality, prevalence and recurrence rates. However, a growing number of studies reported low rate of healthcare-associated CDIs (20% in 2018) among all suspected diarrheal stool tested [10].

More concerns about foods as potential sources of CDI were raised in different countries. The zoonotic transmission of *C. difficile* was hypothesized for more than one decade and was supported by its isolation from food animals and birds including chickens [9,11-17] and the shared PCR ribotypes isolated from them and from humans [18-20]. In addition, the globally increasing incidence of CA-CDI in younger non-hospitalized cases supported the speculation that retail foods can be major sources of this infection [21].

Several foods were studied as potential sources of CDI. Most of the studies reported variable prevalence rates from zero to 7.5% in vegetables, 12.5% in animal meats [9,17,22,23] or at least 50.0% in root vegetables and seafood [24-27]. Carrier rates in birds ranges from zero to 62.0% [5]. Several studies reported *C. difficile* contamination of retail meats of birds and considered it as a potential source of CDI in many populations all over the world [5,9,28,29].

Toxins production by *C. difficile* and its resistance to antibiotics are among the main virulence factors associated with the CDI [30]. It secrets toxin A (enterotoxin; TcdA that increases fluids in the colon with cellular damages), toxin B (cytotoxin; TcdB that increases the cellular damage) and the binary toxin (CDT that augments activities of the other two toxins) [21,31,32]. The prevalence of the ribotype 027, that produces toxins more than other strains by about 10–20 times, was reported to be 14.0% in the USA [33]. A recent study in Japan, reported that 35.0% of *C. difficile* isolates were toxigenic [21]. Non-toxigenic strains can be considered non-pathogenic with a protective effect against colonization by toxigenic ones [34].

Clindamycin, tetracycline and moxifloxacin antibiotics are among the most significant risk antibiotics for developing of CDI (the highest risk was reported with clindamycin) [35]. Moreover, metronidazole and vancomycin were recommended as a treatment of non-severe and severe CDIs, respectively [36]. Recently, metronidazole was recommended as a treatment of non-severe CDIs only if vancomycin and fidaxomicin are unavailable or not tolerated due to the significantly higher recurrence rates associated with metronidazole alone [37]. Fulminant cases need combination of vancomycin with metronidazole [37].

While many reports of *C. difficile* isolation and characterization from retail foods in different countries and populations are available, similar reports are limited in Saudi Arabia. Furthermore, the number of researches performed antibiotics sensitivity testing for *C. difficile* isolates in Saudi Arabia is very limited. As far as I know, this is the first study to evaluate the prevalence of *C. difficile* contamination of retail raw chicken meats in Sakaka, Al-Jouf, Saudi Arabia and characterize the recovered strains regarding their toxigenic activities and antibiotics sensitivity patterns.

Methodology

Study design and samples collection

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 250 raw chicken meat samples (60 from wings, 60 from breasts, 65 from thighs, and 65 from legs) in October and November of the year 2019. The samples were randomly purchased (by simple random sampling procedure; flipping a coin) from 25 shops, markets and supermarkets in Sakaka province, 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

On arrival, the samples were processed using aseptic techniques to avoid their contamination as described elsewhere [38]. In brief, 25 g from each sample was placed into a sterile bag containing sterile phosphate buffered peptone (PBP; 25 mL) and was homogenized for 5 min by hand massaging. One mL from the prepared homogenate was transferred into *C. difficile* Moxalactam Norfloxacin (CDMN) broth (9 mL) (Oxoid, Hampshire, UK) with 0.1% sodium taurocholate and was incubated for 7 days at 37 °C anaerobically by using anaerobic jars, anaerogen kits (Oxoid, Hampshire, UK) and anaerobic indicators (Oxoid, Hampshire, UK). Spore selection was carried out by alcohol shock as the following; CDMN broth culture (1 mL) was mixed with anhydrous ethanol (1 mL), incubated for 1 hour at room temperature, centrifuged at 4,000 rpm for 10 minutes, the supernatant was discarded then sterile swab was used to spread the pellet on the CDMN agar (Oxoid, Hampshire, UK) and the plates were incubated for 72 hours at 37 °C under anaerobic conditions. The suspicious growth on CDMN agar was subcultured into thioglycolate broth followed by 72 hours incubation at 37 °C anaerobically. In addition, the suspicious growth on CDMN agar was subcultured on blood agar. After 72 hours incubation at 37 °C anaerobically, the suspected colonies were tested by standard microbiological and biochemical procedures including testing the odor, colony morphology and Gram stain morphology.

Confirmation of C. difficile

The suspected isolates, those producing horse manure odor with grey white appearance and are Gram-positive bacilli, were tested by L-proline aminopeptidase and *C. difficile* test kits (Oxoid, Hampshire, UK) according to manufacturer's manual. The positive isolates were confirmed by Vitek 2 compact system (BioMérieux, Marcy l'Etoile, France). *C. difficile* ATCC 9689 (Oxoid, Hampshire, UK) was used as a control positive reference strain in all steps [8].

Antimicrobial susceptibility testing

The resistance/susceptibility of *C. difficile* isolates to vancomycin, metronidazole, tetracycline, clindamycin and moxifloxacin antibiotics was detected using Epsilon test (E-test, BioMérieux, Marcy l'Etoile, France) according to the supplier's manual. The Vitek 2-confirmed colonies were spread on brucella agar (Oxoid, Hampshire, UK) supplemented with 5.0% sheep blood and two MIC evaluator strips were placed on the agar then plates were incubated for 72 hours at 37 °C under anaerobic conditions. The minimum inhibitory concentration (MIC) values for vancomycin were compared with the breakpoints established by European committee for antimicrobial susceptibility testing (EUCAST) [39], while the MIC values for metronidazole, tetracycline, clindamycin and moxifloxacin were compared with those defined by Clinical and laboratory standards institute (CLSI) [40]. *C. difficile* ATCC 9689 (Oxoid, Hampshire, UK) was used as a control positive reference strain. Triplicate testing was carried out for each isolate.

Toxin A/B detection

The Xpect CD toxin A/B test (Oxoid, Hampshire, UK) was used to check the confirmed *C. difficile* isolates for toxins A/B production according to manual of the manufacturer. *C. difficile* ATCC 9689 (Oxoid, Hampshire, UK) was used as a control positive reference strain (toxigenic $A^+/B^+/CDT^-$). In brief, the thioglycolate broth of the isolates was incubated for 24 hours at 37 °C under anaerobic conditions. Suitable amount of thioglycolate broth culture was mixed with equal volume of brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) and was incubated for 72 hours at 37 °C under anaerobic conditions. The BHI broth culture was used to detect the A/B toxins [8]. Triplicate testing was carried out for each isolate.

Data analysis

Chi-square and Fisher exact tests were used to compare *C. difficile* prevalence between different chicken meat parts. Statistical significance was considered at $p \leq 0.05$.

Ethics statement

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

Results

The prevalence of *C. difficile* contamination of retail raw chicken meats was screened in 250 samples that were purchased from different shops, markets and supermarkets. Totally, 105 isolates that were rounded, white with a distinctive horse manure smell on CDMN agar were suspected. Fifty of them were positive by L-proline aminopeptidase and *C. difficile* test kits. Eleven isolates (4.4%) were confirmed by Vitek 2 compact system as *C. difficile*. *Clostridium* species other than *C. difficile* were also identified (Table 1). *C. difficile* was isolated from 11/250 (4.40%) chicken samples as the following; 5/65 (7.69%) legs, 3/65 (4.61%) thighs, 2/60 (3.33%) wings and 1/60 (1.67%) breasts ($p = 0.4$).

All Vitek 2 compact system-confirmed *C. difficile* isolates were non-toxigenic for toxins A and B. The E-tests revealed that, although all isolates were vancomycin sensitive, some isolates were intermediate/resistant to metronidazole, tetracycline, clindamycin or moxifloxacin antibiotics with variable degrees (Table 2).

Discussion

Food is not expected to be sterile, for example, contamination of chicken meats with *Campylobacter* and *Salmonella* is expected and common. *C. difficile* production of spores and toxins and its resistance to antibiotics are the main virulence factors associated with CDI. The ability of *C. difficile* to form heat and

Table 1. Identification of isolates by Vitek 2 compact system.

Identification result	Number of isolates
Clostridium difficile	11
Clostridium sordellii	5
Clostridium bifermentans	2
Clostridium septicum	1
Clostridium ramosum	1
Clostridium tertium	1
Clostridium baratii	1
Clostridium glycollicum	1
Non-Clostridium	7
Unidentified	20
Total	50

chemical resistant spores raises alarm because killing these spores during handling, cooking or normal cleaning practices of the kitchen and environmental surfaces may be difficult [41]. Therefore, spore-forming microorganisms might survive in food products even after cooking [42]. Furthermore, Kouassi *et al*. suggested that heating could deplete oxygen in cooked foods, generate anaerobic condition and activate spores to germinate and grow [43]. On the other hand, Weese *et al*. suggested that exposure to low levels of *C. difficile* spores could be a regular event with unclear consequences [44].

C. difficile may contaminate multiple food types with variable contamination rates according to the study performed, country of the study and types of food. Several studies with different methodologies have appeared in response to the increasing interest in the role of *C. difficile* as a foodborne pathogen. It is not easy to understand which methodology is better than the others owing to presence of multiple variables in the reported studies such as number of samples, type of media used during enrichment and culture and incubation duration. In the current study, 50 isolates gave positive reactions with L-proline aminopeptidase and *C. difficile* test kits. However, only 11 isolates were confirmed as *C. difficile* by using Vitek 2 compact system. This can be explained by presence of cross-reaction with other Clostridia as *Clostridium sordellii*, *Clostridium glycollicum* and *Clostridium bifermentans*. As a result, positive reactions should be confirmed by more sensitive and specific method as Vitek 2 compact system with including a particular control positive reference strain of *C. difficile* as ATCC 9689 in each experiment. Some studies used API Rapid ID 32A [45] or Api 20A [43] tests to detect *C. difficile* isolates. Other studies used molecular methods to confirm their results [21,23,32,46]. Generally, some authors predicted higher *C. difficile* isolation rate if the study was performed in winter [47]. In the performed study, the samples, from which *C. difficile* was detected, were collected in October and November months of the year 2019.

The current study in Saudi Arabia provides further evidence that exposure to *C. difficile* from retail chicken meats is not a scarce mood of infection transmission. This was not unexpected considering several studies from different countries reporting detection of *C. difficile* in many food products as ground beef [38], chicken meat [44], raw milk [23] and seafood [23]. Contamination of these foods by *C. difficile* spores may be due to their susceptibility to fecal contamination at their origins and during each stage of their processing.

In the conducted study, low level of chicken meats contamination (4.40%) by *C. difficile* was detected. A recent Slovenian study reported nearly similar contamination level (5.00%) [29]. lower levels of chicken meats contamination were reported from Netherlands [48], Brazil [49] and USA [50] that are 2.70%, 0.00% and 0.00%, respectively. On the other hand, Korean [31], Canadian [44], Turkish [47] and Iranian [51] studies reported higher levels of chicken meats contaminations (16.40%, 12.80%, 8.10% and 15.30%, respectively).

In the current study, all Vitek 2 compact system-confirmed *C. difficile* isolates were non-toxigenic for toxins A and B. The non-toxigenic *C. difficile* strains usually considered non-pathogenic. This result is in agreement with some studies in which 100.00% of *C. difficile* isolates detected in chicken meat samples were non-toxigenic [52]. In addition, some researchers reported predominance of the non-toxigenic *C. difficile* isolates at rates 95.60% and 68.00% [31,47], respectively. On the other hand, some studies reported predominance of the toxigenic *C. difficile isolates* at rates 62.50% and 70.00% [48,51], respectively. Furthermore, some studies reported that 100.00% of the detected *C. difficile* isolates were toxigenic [44,53-55]. Interestingly, in Kuwait, non-toxigenic RT 039/2 was widespread amongst hospitalized patients suffering from diarrhea [56].

Table 2. Antibiotics' minimum inhibitory concentrations of *Clostridioides difficile* isolates by E-tests.

Antibiotics	MIC breakpoints (µg/mL)			MIC values of *C. difficile* isolates and control (µg/mL)												Number of isolates		
	S	I	R	ATCC 9689	Isolate (1)	Isolate (2)	Isolate (3)	Isolate (4)	Isolate (5)	Isolate (6)	Isolate (7)	Isolate (8)	Isolate (9)	Isolate (10)	Isolate (11)	S (%)	I (%)	R (%)
Vancomycin[1]	≤ 2	-	> 2	0.5	1.0	0.25	0.5	1.0	0.5	0.25	0.25	0.5	1.0	1.0	0.5	11 (100)	0 (0)	0 (0)
Metronidazole[2]	≤ 8	16	≥ 32	2.0	0.03	0.03	0.25	4.0	16.0	0.25	0.015	0.12	0.5	0.03	0.06	10 (90.9)	1 (9.1)	0 (0)
Clindamycin[2]	≤ 2	4	≥ 8	1.0	4.0	1.0	0.25	4.0	4.0	0.5	0.25	0.5	2.0	0.25	4.0	7 (63.6)	4 (36.4)	0 (0)
Tetracycline[2]	≤ 4	8	≥ 16	4.0	0.03	0.015	0.06	8.0	0.06	0.25	0.015	0.015	0.06	0.03	8.0	9 (81.9)	2 (18.1)	0 (0)
Moxifloxacin[2]	≤ 2	4	≥ 8	2.0	4.0	0.25	0.25	4.0	8.0	0.5	0.25	1.0	4.0	4.0	8.0	5 (45.5)	4 (36.4)	2 (18.1)

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

Resistance of *C. difficile* to antibiotics is a projecting problem for patients suffering from or at risk of CDI [57]. The *C. difficile* isolates in the conducted study were tested against vancomycin, metronidazole, clindamycin, tetracycline and moxifloxacin antibiotics. Although all isolates were vancomycin sensitive, some isolates were intermediate/resistant to metronidazole, tetracycline, clindamycin or moxifloxacin antibiotics with variable degrees (Table 2). Vancomycin and metronidazole were recommended as a treatment of CDIs [36,37]. Moreover, clindamycin, tetracycline and moxifloxacin are among the most significant risk antibiotics for developing of CDI [35]. The intermediate susceptibility of *C. difficile* to metronidazole is worthy of attention because with oral metronidazole therapy, low level of metronidazole can be achieved in the intestinal lumen (< 0.25 to 9.5 µg/mL) that may not be sufficient to treat *C. difficile* strains with slightly elevated MICs even though they are not clinically resistant according to CLSI criteria.

Many studies reported rare resistance of *C. difficile* to vancomycin and metronidazole [58,59] or even complete susceptibility [28]. Furthermore, A recent research reported scarce *C. difficile* resistance against rifampicin and macrolides (3/80 and 2/80 isolates, respectively) and mild to moderate resistance against moxifloxacin (26/80 isolates) [28]. This is in consistence with result of the current study in which four moxifloxacin-intermediate and two moxifloxacin-resistant isolates out of 11 isolates were detected. The relative decrease in the sensitivity pattern of *C. difficile* to moxifloxacin in comparison with the other four tested antibiotics in the cited study might be due to cross-resistance with other fluoroquinolones that could be used for treatment of many gastrointestinal infections.

The presence of *C. difficile* strains that are non-toxigenic but antibiotic resistant in foods may represent a potential public health risk. Recently, Mooyottu *et al.* detected non-toxigenic-, antibiotic resistant-*C. difficile* in only two pork samples out of 300 meat samples including chicken meats. Furthermore, they detected mobile genetic elements and genes of antibiotic resistance in the two positive samples and warned for formation of multidrug resistant *C. difficile* strains by horizontal genes transfer [52]. Possible gene transfer from some toxigenic and antibiotics-resistant strains to non-toxigenic and antibiotics-sensitive strains, respectively, should be worthy of attention. For example, *C. difficile* clindamycin resistance is usually associated with *erm* genes, that often lie on transposons and are much more likely to be spread between strains.

On the other hand, resistance of *C. difficile* to moxifloxacin is usually associated with point mutation in the quinolone-resistance determining region (QRDR) of *gyr* genes and therefore is unlikely to be spread via horizontal gene transfer.

Multiple researches have revealed that *C. difficile* antibiotic susceptibly/resistance patterns are quite diverse among several countries [37,60]. Consequently, more information about antimicrobial susceptibly/resistance profiles of this fatal organism from different origins is highly needed and important.

Conclusions

As far as I know, this is the first study to evaluate prevalence of *C. difficile* in chicken meats in Al-Jouf, Saudi Arabia. *C. difficile* is an important intestinal pathogen for humans, some food animals and birds including chickens. While its infectious dose is unknown and expected to be variable among persons and populations, it is sensible to predict that lower contamination levels are less dangerous than higher levels. Nevertheless, the low contamination level detected in this study should be of interest to avoid CDI especially for elders, immune-compromised and long terms wide spectrum antibiotics-used persons.

Although the isolated non-toxigenic strains can be considered non-pathogenic with tendency to be sensitive to most of the tested antibiotics, proper use of antimicrobials in poultry industry in Saudi Arabia is essential to decrease the selective drug pressure on *C. difficile* strains associated with chickens. More studies of chickens, chicken meats, animals and their meats with comparison of isolated types with the types isolated from humans in the same locality are required.

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Corresponding author

Ahmed E. Taha, PhD
Microbiology and Immunology unit, Department of Pathology, College of Medicine, Jouf University, Sakaka city, Al-Jouf, Saudi Arabia
Email: aeattia@ju.edu.sa; aeattia@mans.edu.eg; drahmadmicro@yahoo.com

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