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Full Length Research Paper

Investigating the presence of *Clostridium difficile* in vegetables in Jazan markets, Saudi Arabia

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Clostridium difficile **is responsible for** ***C. difficile*** **associated diarrhoea and** ***Pseudomembranous colitis*** **and is currently the most common hospital-acquired infection in the western world. Disease is most often the result of preceding antibiotic use and is traditionally associated with exposure to health-care institutions. The epidemiology of** ***C. difficile*** **in the community is poorly understood. Contaminated faeces from farm animal reservoirs may act as a vehicle for transmission of pathogens to foods. The present study investigated the possible transmission of** ***C. difficile*** **to humans through vegetables. Vegetables were bought from Jazan traditional market. A hundred vegetables were obtained on different occasions, two samples from each, making a total of 200 samples of each vegetable. Samples were tested for the presence of** ***C. difficile*****. Cytotoxicity assay and multiplex PCR were performed on all the positive isolates. Finally, PCR ribotyping was performed on all positive isolates for** ***C. difficile*** **isolates. Three isolates out of 200 samples showed positive for** ***C. difficile*****. All three isolates were positive for** ***tcdA*** **and** ***tcdB*** **toxin genes and were classified as ribotype 078. All three** ***C. difficile*** **isolates showed resistance to Cefotaxime. The present work showed the potential risk of vegetables in transmitting** ***C. difficile*** **to humans.**

Key words: *Clostridium difficile,* infection, vegetable, Jazan.

INTRODUCTION

Clostridium difficile is a Gram-positive, motile, spore-forming, toxin-producing, obligate anaerobe that is present in the natural environment. Over the past decade, it has become a very prominent nosocomial infection worldwide. It is notable that *C. difficile* infection caused ward closures in 5% of UK hospitals in 1993 and, by 1996; this figure had risen to 16% (Popoola et al., 2000). The organism was shown to produce a lethal toxin in experimental animals, but since it was commonly found in the stools of healthy neonates it was classified as 'commensal' and subsequently attracted little attention until 1974, when a comprehensive study showed that *C. difficile* was widespread in nature and could be isolated from the stools of several animal species and from patients' faeces and genitourinary tracts (Hafiz and Oakley, 1976).

C. difficile has been found in a variety of environments, including water, soil, animal feces, and foods (Rodriguez-Palacios, 2007; al Saif and Brazier, 1996) these findings suggest that *C. difficile* may be transmitted to humans through food, although no foodborne cases have been reported. Because ready-to-eat foods have been implicated in foodborne disease outbreaks associated with *Salmonella* species (Sagoo et al., 2003) and *Escherichia coli* O157 (Delaquis et al., 2007), we examined ready-to-eat salads for the presence of *C. difficile*.

We have shown earlier and for the first time that *C. difficile* was present in ready-to-eat salads in Scotland (Bakri et al., 1992). To date, there is no published research that investigates the presence of *C. difficile* in the vegetables in Jazan, Saudi Arabia.

METHODS

50 g sample from each vegetable which were purchased from Jazan traditional market, Jazan, Saudi Arabia was used.

Each sample was homogenized

Table 1. Primers used for PCR toxin genes identifications.

Toxin A gene	Sequence(5'-3')	Amplicon size (bp)
Forward	5'- ATCCGCCTCAACTGGTTATACAAGT -3'	
Reverse	5'- GCTTAGGGGCTTTTACTCCATCAAC -3'	960
Toxin B gene	Sequence(5'-3')	Amplicon size(bp)
Forward	5'-ACTGGATCCACTGGATGGATAT -3'	477
Reverse	5'-ATAAAGCTTTTCACTAATCACTAAT-3'	
16S RNA gene	Sequence(5'-3')	Amplicon size(bp)
Forward	5'-CTCTTGAAACTGGGAGACTTGA-3'	225
Reverse	3'-CCGTCAATTCMTTTRAGTTT-5'	

for 2 mins with 250 ml of Phosphate Buffered Saline (PBS), using a stomacher (BagMixer 400, Interscience Laboratory Inc.). A 1 ml volume of the sample was mixed with 1 ml of ethanol, incubated for an hour at room temperature and then 200 µl was direct plated in duplicate on *C. difficile* agar supplemented with 5% horse blood and *C. difficile* CDA-CDMN (Rodriguez-Palacios et al., 2007). A further 1 ml of bacterial suspension was added to 9 ml of Brain Heart Infusion (BHI)-CDMN, supplemented with selective broth for enrichment culture at 37^{o}C for 7 - 10 days in anaerobic condition, followed by further spore selection by centrifugation and ethanol treatment before plating (Bakri et al., 1992).

PCR amplification of toxin gene sequences

In order to identify the isolates as toxigenic, *C. difficile,* we used three forward and reverse primer sets. One set amplified toxin A sequence (960bp), one set toxin B (477bp), and the last set amplified specifically a *C. difficile* 16S rRNA gene sequence (225bp). Each primer set was run in a separate tube (Table 1).

Reaction volumes of 5 µl resulted from PCR reactions were loaded onto a 1% agarose gel immersed in 1x Tris Acetate-EDTA (TAE) buffer (one litre of 1X TAE buffer was prepared by diluting 1 ml 50X TAE in 1000 ml distilled water) (Sambrook, 2001).

One positive control (6613 strain) was included (positive for toxin A, B, and 16S rRNA genes). To prevent accidental contamination, two negative controls, which were double distilled water, were included.

Multiplex PCR

The materials and methods used here were similar to the methods used in ClabotsCR (Clabots, 1992).

Multiplex PCR for *C. difficile* isolates was carried out for all isolates to detect Toxin A and B genes (*tcdA* and *tcdB*, respectively), the binary toxin genes (*cdtA* and *cdtB*), and 16S rDNA using a procedure based on the method of Persson (Persson et al., 2008). Twelve primers were used for the detection of *tcdA* (629 bp), *tcdB* (410 bp), *cdtA* (221 bp), *cdtB* (262 bp) and 16S rDNA (1026 bp) (Tables 2 and 3). Multiplex PCR method was standard method. The primers were chosen to be specific for *C. difficile* toxins genes and purchased from Promega (USA).

PCR ribotyping

For PCR ribotyping, all isolates and known control strains were grown on Fastidious Anaerobe Agar (FAA) (Oxoid, UK).

PCR ribotyping of *C. difficile* based on intergenic spacer region of 16S-23S rRNA genes of which these are multiple regions sizes in the genome of *C. difficile*. Using a specific primer pair these regions are amplified and separated by molecular size on agarose gel. The resultant banding patterns are analysed by computer. PCR ribotyping of *C. difficile* isolates was carried out using the procedure of Stubbs and co-workers (Stubbs et al., 1999). Oligonucleotide primers (Table 4) were designed to be complementary to the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene.

Then, 20 µl of the concentrated samples were electrophoresed in Metaphor agarose 3% gels (FMC, Rockland, ME) at 150 V for 3 h. As a size marker, a 1000/200bp ladder (Advanced Biotechnologies, UK) was also electrophoresed on the gel. DNA fragments were visualized by staining in ethidium bromide (0.5 mg/ml). Gels were photographed under UV illumination and the

Table 2. The name and sizes of genes targeted in the multiplex PCR.

Gene Target	Primer name	Primer Concentration (µM)	Size of band on gel (bp)
tcdA	tcdA-F3345	0.6	629
	tcdA-R3969	0.6	
tcdB	tcdB-F5670	0.4	410
	tcdB-R6079A	0.2	
	tcdB-R6079B	0.2	
cdtA	cdtA-F739A	0.05	221
	cdtA-F739B	0.05	
	cdtA-R958	0.1	
cdtB	cdtB-F617	0.1	262
	cdtB-R878	0.1	
16s rDNA	PS13	0.05	1062
	PS14	0.05	

Table 3. Primers used for multiplex PCR.

tcdA	**Sequence(5'-3')**	Amplicon size(bp)
TcdA-F3345	5'-GCATGATAAGGCAACTTCAGTGGTA-3'	
TcdA-R3969	5'-AGTTCCTCCTGCTCCATCAAATG-3'	629
tcdB	**Sequence(5'-3')**	Amplicon size(bp)
tcdB-F5670	5'-CCAAARTGGAGTGTTACAAACAGGTG-3'	410
tcdB-R6079A	5'-GCATTTCTCCATTCTCAGCAAAGTA-3'	
tcdB-R6079B	5'-GCATTTCTCCGTTTTCAGCAAAGTA-3'	
cdtA	**Sequence(5'-3')**	Amplicon size(bp)
cdtA-F739A	5'-GGGAAGCACTATATTAAAGCAGAAGC-3'	221
cdtA-F739B	5'-GGGAAACATTATATTAAAGCAGAAGC-3'	
cdtA-R958	5'-CTGGGTTAGGATTATTTACTGGACCA-3'	
ctdB	**Sequence(5'-3')**	Amplicon size(bp)
ctdB-F617	5'-TTGACCCAAAGTTGATGTCTGATTG-3'	262
cdtB-R878	5'-CGGATCTCTTGCTTCAGTCTTTATAG-3'	
16s rDNA	**Sequence(5'-3')**	Amplicon size(bp)
PS13	5'-GGAGGCAGCAGTGGGGAATA-3'	1062
PS14	5'-TGACGGGCGGTGTGTACAAG-3'	

data were saved prior to analysis. The primers were chosen to be specific for *C. difficile* 16S and 23S rRNA genes with no high repetitive areas and purchased from Promega (USA).

Data analysis

Gel data were analysed for PCR-ribotype patterns by comparing with a library of digital gel images formed of each known PCR ribotypes (27 ribotypes) (Anaerobic Reference Laboratory, Scotland). Pattern designations and PCR ribotypes for isolates were assigned by using the Bio Numerics software. This technique performs a high accuracy of comparison between different ribotypes.

Antibiotic susceptibility test

The susceptibility of isolates to antimicrobials was

Table 4. Primers used for Ribotyping.

	Sequence(5'-3')	Amplicon size (bp)
16SrRNA gene	5′-CTGGGGTGAAGTCGTAACAAGG-3′	22
23SrRNA gene	5′-GCGCCCTTTGTAGCTTGACC-3′	20

Table 5. Results for the presence of *C. Difficile* in vegetables samples.

Sample Number	Location	Date of collection	*C. difficile* viable count (cfu/g)
Potato	Jazan	17/03/2016	1.1 x10^2
Potato	Traditional		2.3 x10^2
Parsnip	Market		2 x10^2

determined by estimating the minimum inhibitory concentrations (MICs) of six antibiotics.

The MICs of 6 antimicrobial drugs for these isolates were determined by using E-test strips (AB Biodisk, Solna, Sweden) (Baverud et al., 2003). The following MIC breakpoints were used to define resistance to these drugs: metronidazole, >32 µg/mL; vancomycin, >16 µg/mL; cefotaxime, 64 µg/mL; erythromycin, >8 µg/mL; moxifloxacin, >8 µg/mL; and clindamycin, >8 µg/mL (Clinical and Laboratory Standards Institute, Wayne, PA, USA).

A 48 h bacterial growth suspended in 0.9% saline (Oxoid, UK) and adjusted to no. 1.0 McFarland turbidity standard was inoculated onto FAA plates supplemented with 5% horse blood. FAA was used as standard media for E test and free of any antibiotic. The E test strips of each antibiotic were applied on separate plates onto the agar surface by using sterile forceps, after drying the plates for 15 minutes, and then incubated at 37^{o}C for 24 h in an anaerobic cabinet. The MIC was read as the interception of the elliptical zone of inhibition with the strip.

RESULTS

Examination of samples for *C. difficile*

Of a total of 200 vegetable samples tested for *C. difficile,*three samples were found to be positive after direct plating (Table 5).

Cytotoxicity assay

All the *C. difficile* isolates (three samples) were tested by cytotoxicity assay and were found to be high toxin producers with an end point titer of 10^{-4} as shown in Table 6.

Multiplex PCR

Multiplex PCR for *C. difficile* isolated from vegetable samples showed that all three isolates were toxigenic *C. difficile* and positive for the 16S rRNA gene. All three samples were positive for *tcdA* and *tcdB*but none of the isolates was positive for binary toxin (CDT) as shown in Table 6.

PCR Ribotyping

All isolates were *C. difficile* PCR ribotype 078 as shown in Table 6.

Antibiotic susceptibility testing

The MIC for 6 antibiotics was determined for *C. difficile* isolates. The resistance profile of each *C. difficile* isolate is shown in Table 6. All isolates (ribotypes 078) were susceptible to Metronidazole and Vancomycin. The parsnip isolate was resistant to Moxifloxacin, Clindamycin, Erythromycin and Cefotaxime. Both potato isolates were resistant to Cefotaxime (Table 7).

Discussion

C. difficile infections (CDI) in humans have been a serious concern in recent years, with more community-acquired cases on the increase (Riggs et al., 2007). It was shown in many studies that *C. difficile* can be transmitted through animal feces (Bakri, 2016) and in ready-to-eat salads (Bakri et al., 2009).

It was postulated that farm animals can be a source of infection and their faeces can transmit the microorganism to the vegetables.

Three samples were positive for *C. difficile*. This could imply that the slurry that is used as fertilizer in organic farming may have been the source of *C. difficile* and hence may have acted as the vehicle for *C. difficile* transmission from animal manure to vegetables.

C. difficile has been isolated from soil, water which used for irrigation (Al and Brazier, (1996) or animal manure which is used for fertilizing soil (Hammitt et al., 2007;

Table 6. Molecular typing of vegetables isolates.

Multiplex PCR				PCR Rebotyping	cytotoxicity assay
Samples	***tcd* A/B**	***cdt* A/B**	**16S rDNA**		**Toxin Titre**
Potato	A^+/B^+	-/-	+	078	$_{10}^{-4}$
Potato	A^+/B^+	-/-	+	078	$_{10}^{-4}$
Parsnip	A^-/B^+	-/-	+	078	10^{-4}

Table 7. MIC range of resistance and sensitivity of *C. difficile* isolates to six antimicrobial drugs.

Sample	Ribotype	Metronidazole	Vancomycin	Moxifloxacin	Clindamycin	Erythromycin	Cefotaxme
		ug/ml	ug/ml	ug/ml	ug/ml	ug/ml	ug/ml
Potato	078	S(0.125)	S(0.50)	S(0.70)	I (4.0)	S(1.5)	R (68)
Potato	078	S(0.094)	S (0.38)	S(0.75)	I (6.0)	S (0.75)	R (64)
Parsnip	078	S(0.75)	S (1.0)	R (256)	R (8.0)	R (192)	R (256)

R = Resistance, S = Sensitive, I = Intermediate.

Jhung et al., 2008;Keel and Songer, 2007; Songer and Anderson, 2006)) and any of these could be a source of contamination for the vegetables.

During the last few years, there has been an increasing emphasis on the significance of the consumption of fresh vegetables for the healthy diet (Little et al., 2007). Fresh vegetables are, however, reported to be a principal vehicle in food-borne disease outbreaks (Seymour and Appleton, 2001).

The main sources of pre-processing contamination are manure and faecally-contaminated irrigation water used on the fields and then infected food pickers who are not practicing good hygiene. At the post-harvesting stage, food handlers manually handling this kind of food could also act as a source of contamination.

Farmers must be aware about the possible transmission of *C. difficile* through vegetables to human and therefore taking care about the source of water used for irrigation and manure used for soil.

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