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ORIGINAL ARTICLE

High prevalence of *Clostridium difficile* on retail root vegetables, Western Australia

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Abstract

Aims: The incidence of community-associated *Clostridium difficile* infection (CA-CDI) in Australia has increased since mid-2011. With reports of clinically important *C. difficile* strains being isolated from retail foods in Europe and North America, a foodborne source of *C. difficile* in cases of CA-CDI is a possibility. This study represents the first to investigate the prevalence and genotypes of *C. difficile* in Australian retail vegetables.
Methods and Results: A total of 300 root vegetables grown in Western Australia (WA) were collected from retail stores and farmers' markets. Three vegetables of the same kind bought from the same store/market were treated as one sample. Selective enrichment culture, toxin profiling and PCR ribotyping were performed. *Clostridium difficile* was isolated from 30% (30/100) of pooled vegetable samples, 55·6% of organic potatoes, 50% of nonorganic potatoes, 22·2% of organic beetroots, 5·6% of organic onions and 5·3% of organic carrots. Over half (51·2%, 22/43) the isolates were toxigenic. Many of the ribotypes of *C. difficile* isolated were common among human and Australian animals.
Conclusions: *Clostridium difficile* could be found commonly on retail root vegetables of WA. This may be potential sources for CA-CDI.
Significance and Impact of the Study: This study enhances knowledge of possible sources of *C. difficile* in the Australian community, outside the hospital setting.

Introduction

Clostridium difficile is a well-known cause of healthcare-associated infectious diarrhoea (Slimings *et al.* 2014). Over the past decade, the incidence of community-associated *C. difficile* infection (CA-CDI) has increased worldwide (Freeman *et al.* 2010) and CA-CDI currently accounts for *c.* 26% of all CDI cases in Australia (Slimings *et al.* 2014). Similar changes have been seen in other parts of the developed world (Freeman *et al.* 2010). Recent changes in the global epidemiology of CDI have been mainly attributed to the emergence of so-called 'hypervirulent' strains of *C. difficile*, however, the reasons for an increase in CA-CDI in individuals without the traditional risk factors such as old age, recent hospital stay and antimicrobial exposure remain unclear (Slimings *et al.* 2014).

One possible source of *C. difficile* in the community is food contaminated with *C. difficile*. *C. difficile* ribotype (RT) 078 is the predominant strain found colonizing the gastrointestinal tracts of production animals in the Northern Hemisphere, and this strain has been isolated from retail foods in Canada and the United States (Weese 2010a). Over the last decade, *C. difficile* RT 078 has become a common RT found in human infection in Europe (Bakker *et al.* 2010) and a rising cause of CDI in the United States (Limbago *et al.* 2009). Although some publications report up to 42% prevalence of clinically

important *C. difficile* strains in retail foods (Songer *et al.* 2009), most give much lower prevalence figures, particularly in Europe (Lund and Peck 2015). Nonetheless, it has been hypothesized that CA-CDI could be a foodborne infection (Weese 2010a).

Recent reports have indicated a high rate of gastrointestinal carriage of *C. difficile* in Australian cattle (Knight *et al.* 2013a) and pigs (Knight *et al.* 2015). To date, no study has investigated the prevalence of *C. difficile* in Australian foods although significant contamination of veal calf carcasses has been shown to occur at slaughter (Knight *et al.* 2013a). In this study, we investigated the prevalence of *C. difficile* contaminating Australian-grown root vegetables. Isolates of *C. difficile* were characterized by PCR ribotyping and toxin profiling.

Materials and methods

Sample collection and preparation

A total of 300 root vegetables grown in Western Australia (WA), including organic potatoes (n = 81), organic carrots (n = 57), organic beetroots (n = 54), organic onions (n = 54) and nonorganic potatoes (n = 54), were purchased from 24 retail stores and seven farmers' markets between March 2015 and November 2015. From each retail store and farmers' market, three of each vegetable were purchased depending on availability, except store (W1) where two varieties of organic potatoes were purchased (three each) on the same day. All vegetables were placed into separate bags to prevent cross-contamination. To reduce the possibility of laboratory contamination, the initial preparation of the vegetables was performed before transportation to the laboratory. A vegetable peeler was used to collect potato skin, and a sterile disposable scalpel to harvest the basal plate and roots of onions, and the crown and taproot of carrots and beetroots. Three vegetables of the same kind bought from the same store or market, were then pooled together and treated as one sample. The peeler was soaked with bleach solution (6000 ppm free chlorine) for 1 min between samples as this concentration of bleach and exposure time has been shown to be effective in killing *C. difficile* spores (Hacek *et al.* 2010), and a new scalpel was used between samples for other vegetables. A nonporous tile was used as a chopping board and treated with bleach solution between samples.

Culture, isolation and identification of *C. difficile*

Briefly, vegetable parts and 10 g of potato skin were transferred to 90 ml of BHIB supplemented with 5 g l^{-1} yeast extract, 1 g l^{-1} L-cysteine, 1 g l^{-1} taurocholic acid, 250 mg l^{-1} cycloserine and 8 mg l^{-1} cefoxitin (PathWest Media, Mt Claremont, WA, Australia). A negative control, 10 ml of phosphate-buffered saline added to 90 ml of enrichment broth, was included in each round of sampling to monitor potential contamination. After anaerobic incubation for 10 days, alcohol shock was performed on 2 ml of enrichment broth by the addition of 2 ml of absolute alcohol. After 1 h, the suspension was centrifuged at 3800 ***g*** for 10 min and 10 μl of sediment plated on *C. difficile* ChromID™ agar (BioMérieux, Marcy I' Etoile, France) as described previously (Boseiwaqa *et al.* 2013). Plates were incubated anaerobically in a Don Whitley Scientific Ltd (Otley, Yorkshire, UK) A35 anaerobic chamber with an atmosphere of 10% hydrogen, 10% carbon dioxide and 80% nitrogen, and examined at 24 and 48 h. Ten presumptive *C. difficile* colonies; small, irregular and with a raised umbonate profile, coloured or not (Boseiwaqa *et al.* 2013), were subcultured per ChromID plate onto separate prereduced blood agar plates for identification based on their colony morphology of ground glass appearance, opaque, greyish-white and nonhaemolytic, characteristic chartreuse fluorescence under long-wave UV light (360 nm) and characteristic horse dung odour (Knight *et al.* 2013a). The identity of uncertain isolates was confirmed by the presence of L-proline aminopeptidase activity (Rosco Diagnostica, Tasstrup, Denmark).

Toxin profiling and PCR ribotyping

PCR toxin profiling and ribotyping were performed as previously described (Knight *et al.* 2013a) with slight modification. Briefly, a multiplex PCR was used to detect *tcdA*. The primers included NK2 and NK3 from Kato *et al.* (1991) to detect the A1 region and novel primers (B. Elliott *et al.* unpublished data), tcdA-1 (CAGT-CACTGGATGGAGAATT) and tcdA-2 (AAGGCAA-TAGCGGTATCAG), to detect the A3 region. Each reaction consisted of 4 μl template DNA, 2 mmol l^{-1} $MgCl_2$, 1× buffer II, 0·01% (w/v) BSA, 200 μmol l^{-1} each dNTP, 0·75 U of *Taq* polymerase and 0·2 μmol l^{-1} of each primer in a final volume of 20 μl. The PCR program consisted of 95°C for 10 min then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, then a final extension at 72°C for 7 min. Detection of both *tcdA* regions was required for an isolate to be considered $tcdA^+$. Isolates which did not correspond to any internationally recognized RTs in our library collection of 54 internationally recognized RTs were assigned an internal nomenclature prefixed with QX.

Statistical analysis

Fisher's exact test was performed to compare the prevalence of *C. difficile* on vegetables.

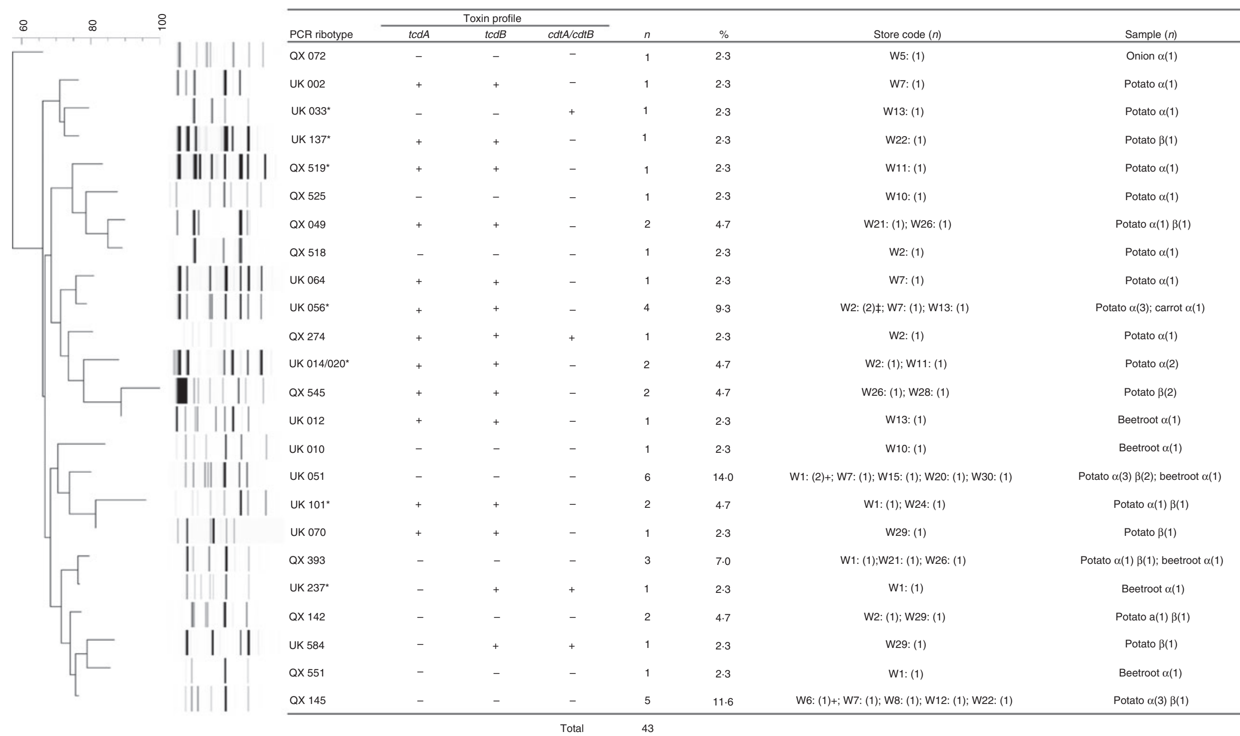
Results

Clostridium difficile was found in 30% of the pooled root vegetable samples; 55·6% (15 of 27 samples) of organic potatoes, 50% (9 of 18) of nonorganic potatoes, 22·2% (4 of 18) of organic beetroots, 5·6% (1 of 18) of organic onions and 5·3% (1 of 19) of organic carrots. There was no significant difference between prevalence in potatoes grown organically and potatoes not grown organically, however, a greater proportion of potato samples was positive for *C. difficile* compared to other vegetables ($P < 0{\cdot}0001$). Of the positive vegetables, 20·0% (3 of 15) of organic potatoes, 22·2% (two of nine) of nonorganic potatoes and 25·0% (one of four) of organic beetroots were contaminated with more than one *C. difficile* ribotype.

Twenty-four RTs of *C. difficile* were identified (Fig. 1), 13 of which were internationally recognized RTs. Half of the isolated strains were toxigenic (51·2%, 22 of 43), predominantly A+B+CDT− (81·8%, 18 of 22). Nontoxigenic strains comprised 48·8% (21 of 43) of isolates. *Clostridium difficile* RT QX 274 was the only isolate with toxin profile A+B+CDT+. RT 027 and RT 078 strains were not found. The most common RT was nontoxigenic RT 051 which comprised 14·0% (6 of 43) of isolates. The next most prevalent RTs were QX 145 (11·6%), 056 (9·3%), QX 393 (7·0%), 014/020 (4·7%), 101 (4·7%), QX 049 (4·7%), QX 142 (4·7%) and QX 545 (4·7%; Fig. 1).

Discussion

The prevalence of *C. difficile* contamination of root vegetables in the present study was higher than reported in other countries, ranging from at least 10% (30 of 300 vegetables) to 30% (90 of 300 vegetables) due to pooling of three vegetables into a single sample. In France, 2·9% (3 of 104) of ready-to-eat salads and pea sprouts were positive for *C. difficile* (Eckert *et al.* 2013); while 4·5% (5 of 111) and 7·5% (3 of 40) of retail vegetables in Canada (Metcalf *et al.* 2010) and Scotland (Bakri *et al.* 2009), respectively, were contaminated with *C. difficile*. The higher prevalence in the present study may be due to a number of factors. First, this was a study of root vegetables while other studies were mainly of leaf vegetables or



PCR ribotype	Toxin profile *tcdA*	Toxin profile *tcdB*	Toxin profile *cdtA/cdtB*	*n*	%	Store code (*n*)	Sample (*n*)
QX 072	−	−	−	1	2·3	W5: (1)	Onion α(1)
UK 002	+	+	−	1	2·3	W7: (1)	Potato α(1)
UK 033*	−	−	+	1	2·3	W13: (1)	Potato α(1)
UK 137*	+	+	−	1	2·3	W22: (1)	Potato β(1)
QX 519*	+	+	−	1	2·3	W11: (1)	Potato α(1)
QX 525	−	−	−	1	2·3	W10: (1)	Potato α(1)
QX 049	+	+	−	2	4·7	W21: (1); W26: (1)	Potato α(1) β(1)
QX 518	−	−	−	1	2·3	W2: (1)	Potato α(1)
UK 064	+	+	−	1	2·3	W7: (1)	Potato α(1)
UK 056*	+	+	−	4	9·3	W2: (2)‡; W7: (1); W13: (1)	Potato α(3); carrot α(1)
QX 274	+	+	+	1	2·3	W2: (1)	Potato α(1)
UK 014/020*	+	+	−	2	4·7	W2: (1); W11: (1)	Potato α(2)
QX 545	+	+	−	2	4·7	W26: (1); W28: (1)	Potato β(2)
UK 012	+	+	−	1	2·3	W13: (1)	Beetroot α(1)
UK 010	−	−	−	1	2·3	W10: (1)	Beetroot α(1)
UK 051	−	−	−	6	14·0	W1: (2)+; W7: (1); W15: (1); W20: (1); W30: (1)	Potato α(3) β(2); beetroot α(1)
UK 101*	+	+	−	2	4·7	W1: (1); W24: (1)	Potato α(1) β(1)
UK 070	+	+	−	1	2·3	W29: (1)	Potato β(1)
QX 393	−	−	−	3	7·0	W1: (1);W21: (1); W26: (1)	Potato α(1) β(1); beetroot α(1)
UK 237*	−	+	+	1	2·3	W1: (1)	Beetroot α(1)
QX 142	−	−	−	2	4·7	W2: (1); W29: (1)	Potato a(1) β(1)
UK 584	−	+	+	1	2·3	W29: (1)	Potato β(1)
QX 551	−	−	−	1	2·3	W1: (1)	Beetroot α(1)
QX 145	−	−	−	5	11·6	W6: (1)+; W7: (1); W8: (1); W12: (1); W22: (1)	Potato α(3) β(1)
			Total	43			

Figure 1 *Clostridium difficile* PCR ribotypes and toxin gene profiles isolated from Western Australian vegetables. PCR ribotype pattern analysis was performed by creating a neighbour-joining tree, using the Pearson correlation (optimization, 5%; curve smoothing, 1%). **Clostridium difficile* PCR ribotypes common in humans and/or Australian production animals; α, organic; β, nonorganic; †, two varieties of organic potatoes (purchased from W1 on the same day in October 2015) were positive for UK 051; ‡a sample of organic carrots (purchased in March 2015) and organic potatoes (purchased in August 2015) from W2 were positive for *C. difficile* UK 056. Isolates which did not correspond to any internationally recognized RTs in our library collection were given an internal nomenclature prefixed with QX.

vegetables cultivated above ground. Root vegetables are likely to have more soil residue on their surfaces and this may have contributed to the high prevalence, as *C. difficile* can be abundant in agricultural soil (AlSaif and Brazier 1996; Simango 2006). With potatoes, for example, sampling a greater surface area covered in soil may have led to better detection of *C. difficile* contamination. The culture method used is likely to have been more sensitive as three vegetables of the same kind were pooled and treated as one sample, so as little as one *C. difficile*-positive vegetable would result in a positive sample. However, even if that was the case, the prevalence reported in this study would still be higher (≥10%) compared to other studies. The present study also used a bigger volume of enrichment broth compared to other studies, as well as a longer incubation time (Bakri *et al.* 2009; Metcalf *et al.* 2010; Eckert *et al.* 2013). Currently, there are no international standards for isolating *C. difficile* from food, although the method used was similar to the US CDC-recommended method for meat (Limbago *et al.* 2012) except for the longer incubation time.

It is also possible that the higher prevalence of *C. difficile* on vegetables reflects different vegetable growing and processing practices in Australia which may have resulted in contamination of produce by animal manure. In Australia, *c.* 1·7 million tonnes per year of animal manure is applied to agricultural land as fertilizer, 6·8% (115 989 t) of which is used for farming in WA (Australian Bureau of Statistics 2011). This includes manure from chicken, cattle, pig and sheep farms. In this study, many of the *C. difficile* RTs identified on the vegetables are commonly found in Australian production animals. Of the RTs represented among the 43 food isolates (Fig. 1), *C. difficile* RT 014/020 was also the most common strain in Australian piglets (23·4%) in a nationwide surveillance study (Knight *et al.* 2015). In addition, RT 014/020 was the most prevalent strain in human clinical samples in WA between October 2011 and September 2012, accounting for 30% (99 of 330) of CDI cases (Cheng *et al.* 2016). *Clostridium difficile* RT 056, which constituted 9·3% (4 of 43) of the food isolates, was the fourth most prevalent strain in humans (3·9%; 13 of 330; Cheng *et al.* 2016) and the third most prevalent strain in Australian veal calves (7·7%; 16 of 209; Knight *et al.* 2013a). Other *C. difficile* RTs isolated with an epidemiological link to Australian livestock include RTs 101, 137, 033 and 237. Ribotypes 101 (40%; 6 of 15) and 137 (13·3%; 2 of 15) were the two most prevalent RTs in lambs (Knight and Riley 2013b), RT 033 was the second most prevalent ribotype in both veal calves (19·6%; 41 of 209; Knight *et al.* 2013a) and piglets (13%; 20/154; Knight *et al.* 2015), while RT 237 is a pig strain unique to one piggery in WA (Knight *et al.* 2015). All of these RTs of *C. difficile* have been isolated from human cases of CDI in Australia (Androga *et al.* 2015; Furuya-Kanamori *et al.* 2016; McGovern *et al.* 2016).

Neither *C. difficile* RT 027, RT 078 nor RT244 were found in this study. It is not surprising that RT 027 was not isolated as it has been isolated infrequently in Australia (Richards *et al.* 2011) and RT 078 has never been isolated from an Australian production animal (Knight and Riley 2013b; Knight *et al.* 2013a, 2015). RT244 emerged in Australia in 2011/2012 (Eyre *et al.* 2015) as a cause of severe community-acquired infection and the pattern of disease across Australia suggested a foodborne outbreak. During this period RT244 was the third most common RT of *C. difficile* detected in Australia (Huber *et al.* 2014). It has subsequently declined to undetectable levels implying that there is no longer a source or reservoir in Australia. However, the isolation of other clinically important *C. difficile* strains from retail vegetables in WA suggests ongoing foodborne transmission of CDI is likely. With many of the isolated RTs being common in animals, the most likely source of contamination is through the use of animal manure as fertilizer in agricultural farming. However, there are other possible points of contamination including downstream food processing, at wholesale, during transport and at the retail market.

Laboratory contamination is unlikely to be responsible for the high prevalence of *C. difficile* in the present study as all samples were prepared using aseptic techniques prior to transportation to the laboratory for enrichment culture, and a diverse range of *C. difficile* PCR RTs was isolated including RTs novel to our laboratory, QX 518 (A−B−CDT−), QX 519 (A+B+CDT−), QX 525 (A−B−CDT−), QX 545 (A+B+CDT−) and QX 551 (A−B−CDT−). Such diversity is highly unlikely to represent laboratory contamination.

This study has several limitations. Firstly, the level of *C. difficile* contamination on the vegetables was not determined. The concentration of *C. difficile* spores on contaminated foods is generally presumed to be low, with studies that report positive samples detecting *C. difficile* by enrichment culture only (Weese *et al.* 2010b) or, if positive by direct culture, counts of 20–240 spores per g (Weese *et al.* 2009). The infective dose of *C. difficile* is currently unknown, although suggestion has been made that it could be low (100–1000 spores) depending on host susceptibility (Hensgens *et al.* 2012; Warriner *et al.* 2017). Secondly, some strains were given internal nomenclature (QX types) because they did not correspond to any internationally recognized ribotypes in our library collection. Thus, making them impossible to compare with RTs reported in other studies. Another limitation of this study was that all the vegetables tested were grown in WA. For a more thorough understanding of the

prevalence and molecular types of *C. difficile* on retail foods in Australia, larger studies involving samples from multiple States and Territories are necessary. A more structured sampling regime could identify any potential seasonality.

This study represents the first to determine the prevalence of *C. difficile* in Australian retail foods. A higher prevalence of *C. difficile* was found on retail vegetables in WA (ranging from ≥10–30%) in comparison to reports from Europe and North America. The finding of diverse *C. difficile* RTs common to livestock and humans suggests that CDI, especially CA-CDI, might have a foodborne transmission component in Australia and that the strains of *C. difficile* involved may be of animal origins. However, whether transmission is via food per se, or food contaminates the environment (e.g. a home kitchen), remains to be determined. To show foodborne transmission of CDI, further studies with more discriminatory typing methods, such as whole-genome sequencing, are necessary to compare the relatedness of these food isolates with those of human CDI.

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Conflict of Interest

No conflict of interest declared.

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