Contamination of ready-to-eat raw vegetables with 
Clostridium difficile in France

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The presence of Clostridium difficile in food like shellfish, vegetables and meat has been reported in several publications during the past few years. The objective of this study was to assess the prevalence of ready-to-eat raw vegetables contaminated with C. difficile in France. One hundred and four ready-to-eat salads and vegetables were studied. Toxigenic C. difficile strains were isolated in three samples (2.9%): two ready-to-eat salads (one heart of lettuce and one lamb’s lettuce salad) and one portion of pea sprouts. The strains belonged to three different PCR ribotypes: 001, 014/020/077 and 015. The detection thresholds for vegetative cells and spores cells varied between 1 and 3 c.f.u. in 20 g salad and between 6 and 15 c.f.u. in 20 g salad, respectively, for the method employed.

INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming, anaerobic bacterium responsible for colitis and antibiotic-associated diarrhoea. The main risk factors for C. difficile infection (CDI) include age (>65 years), antibiotic exposure and previous hospitalization. In healthcare settings, C. difficile transmission occurs via the healthcare worker’s hands or the environment. In the last decade, many important outbreaks of CDI have been described worldwide and the incidence has increased in many countries (Kuijper et al., 2006; Loo et al., 2005). This trend is assumed to be due in part to the emergence and rapid spread of the new hypervirulent 027/NAP1 clone (McDonald et al., 2005).

Other changes have also occurred in the epidemiology of CDI. For example, community cases of CDI have increased and have been described in people previously considered to be at low risk (young healthy individuals or individuals with no previous hospitalization and no recent exposure to antibiotics), raising questions about the source of contamination in this specific population (Hensgens et al., 2012; Wilcox et al., 2008). PCR ribotype 078, 001 and 014 strains have been identified in community-acquired cases (Bauer et al., 2009; Goorhuis et al., 2008a; Wilcox et al., 2008).

C. difficile has been recognized recently as a pathogen or commensal in numerous animals, such as pigs, cattle, foals, horses, calves and poultry, and also in household pets (Avbersek et al., 2009; Riley et al., 1991). PCR ribotype 078 and/or toxinoftype V strains are the predominant strains in calves and pigs (Goorhuis et al., 2008b; Keel et al., 2007; Rupnik et al., 2008). Interestingly, there is an overlap between PCR ribotypes found in humans and animals (Debast et al., 2009), raising the question of interspecies transmission and possible routes for exposure to CDI in humans.

Recent changes in C. difficile epidemiology have led the scientific community to explore the possible role of food as a vector for C. difficile in humans. Detection of C. difficile in food products has been reported recently in shellfish, vegetables and meat in several publications (Bakri et al., 2009; Bouttier et al., 2010; Hensgens et al., 2012; Metcalf et al., 2010). Contamination rates varied between North America where a high prevalence of C. difficile was found and Europe where a lower prevalence has been reported (Hensgens et al., 2012). Few data are available in France. The objective of this study was to assess the prevalence of ready-to-eat raw vegetables contaminated with C. difficile in France.

METHODS

Sixty ready-to-eat salads and 44 ready-to-eat raw vegetables (carrots, mushrooms, radishes, broccoli, celery roots, red cabbage, cauliflower, soya bean, pea sprouts and beets) were purchased from five retail stores and supermarkets in Paris and surrounding areas between September 2010 and March 2011. These vegetables came from 19 different manufacturers. Twenty grams of each sample was crushed in 75 ml pre-reduced brain–heart infusion (BHI) broth (BD) supplemented with 8 µg cefoxitin ml−1, 250 µg cycloserine ml−1 and 0.1% taurocholate. The broth was incubated for 72 h under anaerobic conditions, and tenfold serial dilutions of the BHI broth were plated on TCCA (52 g BHI agar l−1 supplemented with 5% defibrinated horse blood, 0.1% taurocholate 100 µl, 250 µg cycloserine ml−1 and 8 µg cefoxitin ml−1 plated on TCCA). The BHI broth was also plated on TCCA after

Abbreviation: CDI, Clostridium difficile infection.
alcohol shock (v/v, incubation 30 min). Suspected colonies were identified by conventional methods (Gram staining, odour and bioMérieux Rapid ID32A strips).

DNA from colonies was extracted with an InstaGene Matrix kit (Bio-Rad). Genes for the large clostridial toxins A and B (cdaA and cdbB) were detected by PCR. Strains were characterized by PCR ribotyping as described previously (Bidet et al., 1999). PCR ribotype profiles were analysed using PFQuest (Bio-Rad). Characterization of tcdC (deletion of 18, 39 or 54 bp, or no deletion) was also investigated using primers P1367 (5'-ATTCTCAAAAACAGAAATAGAAATTT-3') and P1368 (5'-CTCTCACCATCTCCTAATACTT-3'). PCR to detect binary toxin genes was performed using primers P368 (5'-GAAGCAGAAGAAATAGAGC-3') and P369 (5'-GGTTTTTCATCACCTTTTCCAGG-3') to detect cdtA and P304 (5'-TAAAACGAGAATCTGC-3') and P590 (5'-TTCCTAAATTTGGTTCTTTCCAGC-3') to detect cdtB. Susceptibility to metronidazole and vancomycin was determined by conventional methods (Gram staining, odour and alcohol shock (v/v, incubation 30 min). Suspected colonies were identified by conventional methods (Gram staining, odour and bioMérieux Rapid ID32A strips).

For determination of the detection threshold of the method, serial dilutions of (i) vegetative cells of C. difficile strain VP110463 (PCR ribotype 087) obtained from a BHI culture incubated for 24 h or (ii) spores of C. difficile strain CD196 (PCR ribotype 027) were prepared in distilled water. Each dilution (100 μl, ranging from 10^-1 to 10^-6) was inoculated onto TCCA plates and incubated for 48 h in an anaerobic atmosphere. Simultaneously, 20 g of C. difficile-negative spores was prepared with 200 μl of different dilutions of C. difficile suspensions. The spiked colonies were processed as described above and incubated for 72 h at 37°C in anaerobic atmosphere. To obtain spores of C. difficile, 5 ml of a 7-day culture of CD196 in BHI broth was mixed with 5 ml ethanol for 30 min. After centrifugation at 3800 g for 10 min, the pellet was resuspended in 1 ml BHI broth. Experiments were performed in duplicate.

RESULTS AND DISCUSSION

The detection thresholds for vegetative cells and spores varied between 1 and 3 c.f.u. in 20 g salad and between 6 and 15 c.f.u. in 20 g salad, respectively (Table 1). None of the four studies that previously focused on C. difficile in vegetables evaluated the detection threshold of their methods. As a result, comparison among studies is difficult (Al Saif & Brazier, 1996; Bakri et al., 2009; Metcalf et al., 2010; Zidaric & Rupnik, 2012). To compare contamination rates between products and countries, standardization of the methods to detect C. difficile in food is necessary.

Toxicogenic C. difficile strains were isolated in three samples (2.9%): two ready-to-eat salads [one heart of lettuce (CRU38) and one lamb’s lettuce salad (CRU24)] and one portion of pea sprouts (CRU87) were positive for C. difficile. The three strains were negative for binary toxin, but truncated forms of the cdaA and cdbB genes were detected. No deletion was found in tcdC. The three strains were all susceptible to metronidazole and vancomycin. The strains belonged to three different PCR ribotypes: 001 (CRU24), 014/020/077 (CRU38) and 015 (CRU87). Interestingly, the PCR ribotypes isolated in this study (001, 014/020/077 and 015) are the major PCR ribotypes common in France and, along with PCR ribotype 078/126, represent the three most prevalent PCR ribotypes in France (Eckert et al., 2013). Food contamination in the laboratory remains a possibility, but, although PCR ribotypes 014/020/077 and 015 are common in France, PCR ribotype 001 is isolated less frequently and therefore its presence in salad is unlikely to be due to contamination in our laboratory.

To date, only a few studies have focused on C. difficile in vegetables and salads. Al Saif & Brazier (1996) analysed 300 raw vegetables on sale in retail premises in Cardiff by direct plating and found a 2.4% contamination rate. Among the seven C. difficile strains isolated, five produced toxin A. Unfortunately, no data were available regarding strain typing. In Scotland, Bakri et al. (2009) found that 7.5% (3/40) of ready-to-eat salads were positive for spores: one strain belonged to PCR ribotype 001 and the other two to PCR ribotype 017. Interestingly, the contaminated salads were imported from EU countries. In a third study conducted in Canada, 4.5% of vegetables were contaminated (Metcalf et al., 2010). Among the five strains isolated, four were recovered from vegetables imported from China. Three strains were of PCR ribotype 078, whilst the other two were of toxino-type 0 and of a PCR ribotype involved in CDI in humans. In a recent study performed in Slovenia, C. difficile was not detected in any of the eight ready-to-eat salads and sprouts tested, but the number of samples was limited (Zidaric & Rupnik, 2012). C. difficile has been found in ready-to-eat raw vegetables in France and the prevalence observed was quite similar to that reported previously in South Wales and Canada but lower than that reported in Scotland (Al Saif & Brazier, 1996; Bakri et al., 2009; Metcalf et al., 2010). However, differences in prevalence may reflect heterogeneity in procedures for sample processing.

One limitation of this study was that we were unable to quantify the level of contamination by C. difficile in ready-to-eat vegetables. It is assumed that it is mainly spores that are recovered from food, but we were unable to distinguish between spores and vegetative cells in this study because of the method used. Enumeration of spores in food is likely to be an important factor to determine, even if the infectious dose in humans is unknown and probably varies from one individual to another, depending on many factors such as age, immunity, underlying disease and antibiotic exposure. C. difficile in food may be a potential route of community-acquired CDI. However, no food-borne transmission has been clearly established and no outbreak related to food has been reported so far (Hensgens et al., 2012).

Conclusion

C. difficile is well known as a human pathogen responsible for CDI, but this bacterium is also responsible for animal infection and can be found in different food products including raw vegetables. This presence may reflect contamination of the environment (soil) or may be
occuring during the processing step. Contamination of food by *Clostridium difficile* may raise concerns for public health, and more studies are needed to evaluate the impact of food contamination in community cases of CDI.

**REFERENCES**


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**Table 1. Detection thresholds for vegetative cells and spores**

The detection thresholds are indicated in bold. + = presence of *Clostridium difficile* colonies.

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<tr>
<th>Experiment</th>
<th>Dilution</th>
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<td><strong>Vegetative cells: experiment 1</strong></td>
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<tr>
<td>Broth enumeration of vegetative cells from VPI 10463 [c.f.u. (100 µL)$^{-1}$]</td>
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<td>Results of salads (20 g) spiked with 100 µl of each broth dilution</td>
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<td><strong>Vegetative cells: experiment 2</strong></td>
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<td>Broth enumeration of vegetative cells from VPI 10463 [c.f.u. (100 µL)$^{-1}$]</td>
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<td>Results of salads (20 g) spiked with 100 µl each broth dilution</td>
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<td><strong>Spores: experiment 1</strong></td>
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<td>Broth enumeration of spores from CD 196 [c.f.u. (100 µL)$^{-1}$]</td>
<td>&gt;300</td>
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<td>Results of salads (20 g) spiked with 100 µl each broth dilution</td>
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<td><strong>Spores: experiment 2</strong></td>
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