Clostridioides difficile (including epidemiology)

Prevalence and characterization of Clostridioides difficile isolates from retail food products (vegetables and meats) in Japan

Masaru Usui a,*, Aika Maruko a, Michiko Harada a, Fumi Kawabata a, Tsubasa Sudo a, Sayo Noto a, Toyotaka Sato b, Masaaki Shinagawa c, Satoshi Takahashi c, d, Yutaka Tamura a

a Laboratory of Food Microbiology and Food Safety, Department of Health and Environmental Sciences, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
b Department of Microbiology, Sapporo Medical University School of Medicine, Sapporo, Japan
c Division of Laboratory Medicine, Sapporo Medical University Hospital, Sapporo, Japan
d Department of Infection Control and Laboratory Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

A R T I C L E   I N F O

Article history:
Received 9 October 2019
Received in revised form 19 November 2019
Accepted 25 November 2019
Available online 26 November 2019
Handling Editor: Vincent Rotimi

Keywords:
Clostridioides difficile
Meat
Retail food
Ribotype 014
Toxinotype XIb
Vegetables

A B S T R A C T

The present study aimed to elucidate the prevalence of Clostridioides difficile in Japanese retail food products. For this purpose, retail food samples (242 fresh vegetables and 266 retail meat samples: 89 chicken meat; 28 chicken liver; 200 pork meat; 24 pig liver; 127 beef meat) were collected from 14 supermarkets between 2015 and 2019. C. difficile was isolated from eight (3.3%) fresh vegetable, six (6.7%) chicken meat, one (3.6%) chicken liver, one (0.5%) pork meat, and two (1.6%) beef meat samples; it was not isolated from pig liver. Of these isolates, 35% were toxigenic. All isolates were typable by PCR ribotyping and were resolved into 12 PCR ribotypes. Among these isolates, ribotype 014, which is distributed worldwide including in Japanese clinical cases, was detected among vegetable isolates. Therefore, although the C. difficile contamination rate in Japanese retail foods was low, these sources can be contaminated and could transmit these bacteria to humans.

© 2019 Elsevier Ltd. All rights reserved.

1. Introduction

Clostridioides (Clostridium) difficile causes antibiotic-associated diarrhea and pseudomembranous colitis in humans. According to a report published by the Centers for Disease Control and Prevention, C. difficile is the most important antimicrobial-resistant threat to public health in the United States [1]. Although the incidence of C. difficile infection (CDI) in Japan is lower than that in the United States and European countries, this infection remains a genuine problem in Japan as well [2–4].

Virulent strains of C. difficile produce two large clostridial toxins, toxins A (TcdA) and B (TcdB), encoded by the genes tcdA and tcdB, respectively [5]. TcdA binds the apical side of host enterocytes, whereas TcdB binds the basolateral side of these cells. Both toxins are proteolytic and are taken into the cytoplasm of cells. In addition, some virulent strains of C. difficile produce a third toxin, known as binary toxin (CDT).

Recently, the incidence of community-associated C. difficile infection (CA-CDI) in younger patients without a previous history of hospitalization or antibiotic treatment has been increasing globally [6,7]. This is due to the increased frequency of CA-CDI in patients and the speculation that C. difficile from retail foods (both meats and vegetables) causes these infections in humans [8,9]. In previous studies, C. difficile has been isolated from retail meats [7,8,10,11]. In addition, the previous study showed that C. difficile, derived from livestock manure compost, could be transferred to vegetables [12]. Moreover, a commonality was seen in cases where C. difficile was isolated from vegetables in several countries [10,13–16], suggesting that retail foods can serve as a potential source of C. difficile for humans.

The incidence of CA-CDI in Japan is relatively low compared to that in the United States and European countries; additionally, CA-
CDI is usually not severe [17]. Moreover, the major circulating strains among CDI in Japan differ from those in other countries [18]. Whereas there are several reports of *C. difficile* isolation from foods in the USA, European countries, and Australia [7,10,11,13], there are no reports of *C. difficile* isolation from retail foods in Japan. Therefore, prevalence analysis and characterization of *C. difficile* from retail foods in Japan are required. The present study aimed to determine the prevalence of *C. difficile* from retail foods (vegetables and meats) in Japan. To this end, we isolated *C. difficile* from retail foods and characterized these isolates.

2. Materials and methods

2.1. Bacterial strains and retail food samples

A total of 242 fresh vegetables were continuously purchased from seven supermarkets in the Hokkaido prefecture between March 2015 and May 2017 (Table 1). Samples consisted of 170 root vegetables (39 burdock, 39 taro, 39 ginger, 19 onions, 13 ginseng, 11 green onions, seven radish, two yam, and one wasabi), 41 leaf vegetables (14 lettuce, nine cabbages, six Chinese cabbages, six Chinese chive, three cut cabbages, one broccoli, one asparagus, and one pickled scallion), 27 fruit vegetables (14 cucumbers, 11 tomatoes, and two petit tomatoes), and four water culture vegetables (two bean sprouts, one bean seeding, and one sprout broccoli). A total of 468 retail meat samples were purchased from 14 supermarkets in the Hokkaido prefecture between March 2015 and March 2016 and March 2018 to March 2019 (Table 1). Samples included 89 chicken meat, 28 chicken liver, 200 pork meat, 24 pig liver, and 127 beef meat samples. These samples were purchased once per month; all samples were domestic products, stored at 4 °C, and examined within 4 days of collection.

To measure genetic relatedness, American Type Culture Collection (ATCC) 9689 (Ribotype 001), 43393 (Ribotype 060), 700057 (Ribotype 038), BAA-1870 (Ribotype 027), and BAA-1875 (Ribotype 078) were used as reference strains. The DNA extracts of ribotype strains (G5S-01 = Ribotype 017, TRI15 = Ribotype 369, JND13-004 = Ribotype 001, KGH20 = Ribotype 002, JND08-035 = Ribotype014, JND11-059 = Ribotype 018, and OG39 = Ribotype 046) were kindly provided by Haru Kato (National Institute of Infectious Diseases) and used as references to determine the PCR ribotype.

2.2. Isolation and identification from retail foods

*C. difficile* strains were isolated by an enrichment cultivating method previously reported [19], with some modifications. *C. difficile* was isolated using *C. difficile* culture agar (CM601, Oxoid, Basingstoke, United Kingdom) supplemented with *C. difficile* moxalactam norfl Roxacin (CDMN, SR0173E; Oxoid) and 5% horse blood (SR0048C; Oxoid). *C. difficile* enrichment broth was prepared by mixing the ingredients of CM6001, except for the agar, with 0.1% sodium taurocholate. To this, 5 g of each sample was homogenized and added to 20 ml of the *C. difficile* enrichment broth and incubated anaerobically at 37 °C for 15 days. Alcohol shock for spore selection was performed by mixing 2 ml homogenized culture broth with 99.5% ethanol (1:1 [v/v]) for 50 min. After centrifugation (3,800×g for 10 min), the sediment was streaked onto CCMA-Ex agar (Nissui Pharmaceutical, Tokyo, Japan) and was incubated anaerobically at 37 °C for 48 h. For individual samples, a maximum of three colonies were identified as *C. difficile* based on colony morphology and then selected for further analysis.

For the identification of *C. difficile*, polymerase chain reaction (PCR) was performed. DNA was extracted from isolates using InstaGene Matrix (BioRad, Hercules, CA, USA), according to the manufacturer’s instructions. Three regions with specific sequences of *C. difficile* were amplified using primers specific to three target regions [20–22].

2.3. Toxicity

The presence of toxin A, B, and the binary toxin was tested by multiplex PCR as previously described [23] to amplify *tcdA, tcdB*, and *cdtA/B*, respectively. Toxigenic isolates were toxinotyped by PCR-RFLP as previously described [24].

2.4. PCR ribotyping

Isolates were ribotyped by PCR as described, with some modifications [21]. PCR reactions were scaled down to 50 μl, and amplified PCR products were concentrated to approximately 10 μl by heating at 75 °C for 90–120 min. Subsequently, reactions were electrophoresed for 4 h at 120 V on 3% Metaphor agarose (Lonza Rockland Inc., Basel, Switzerland). Banding patterns were analyzed with BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Similarity and diversity were assessed by applying the Dice coefficient, and strains were clustered based on the unweighted pair group method with arithmetic means. Several types of PCR ribotypes were used as a control; if matching with these known ribotypes was observed, the PCR ribotype of the isolates could be determined.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Years</th>
<th>Sample No.</th>
<th>Isolated samples</th>
<th>Isolated clones</th>
<th>Ribotype</th>
<th>Toxigenic clones</th>
<th>Toxinotype^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root vegetable</td>
<td>2015–2017</td>
<td>170</td>
<td>8 (4.7%)</td>
<td>9</td>
<td>R6 (3), O14 (1), F2 (1), F3 (1), F4 (1), F5 (1), F6 (1)</td>
<td>4 (44.4%)</td>
<td>Xlb (3), XIII (1)</td>
</tr>
<tr>
<td>Leaf vegetable</td>
<td>2015–2017</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>F1 (1)</td>
<td>0</td>
<td>V (1)</td>
</tr>
<tr>
<td>Fruit vegetable</td>
<td>2015–2017</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>F1 (1)</td>
<td>0</td>
<td>V (1)</td>
</tr>
<tr>
<td>Water culture</td>
<td>2015–2017</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>F6 (2)</td>
<td>2 (100%)</td>
<td>Xlb (2)</td>
</tr>
<tr>
<td>Subtotal Vegetables</td>
<td>2015–2017</td>
<td>242</td>
<td>8 (3.3%)</td>
<td>9</td>
<td>R6 (3), O14 (1), F2 (1), F3 (1), F4 (1), F5 (1), F6 (1)</td>
<td>4 (44.4%)</td>
<td>Xlb (3), XIII (1)</td>
</tr>
<tr>
<td>Chicken meat</td>
<td>2015–2016</td>
<td>89</td>
<td>6 (6.7%)</td>
<td>7</td>
<td>F1 (4), F7 (1), F8 (1), F9 (1)</td>
<td>1 (14.3%)</td>
<td>V (1)</td>
</tr>
<tr>
<td>Chicken liver</td>
<td>2015–2016</td>
<td>28</td>
<td>1 (3.6%)</td>
<td>1</td>
<td>F1 (1)</td>
<td>0</td>
<td>V (1)</td>
</tr>
<tr>
<td>Pork meat</td>
<td>2015–2019</td>
<td>200</td>
<td>1 (0.5%)</td>
<td>1</td>
<td>F10 (1)</td>
<td>0</td>
<td>V (1)</td>
</tr>
<tr>
<td>Pig liver</td>
<td>2015–2016</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>F1 (1)</td>
<td>0</td>
<td>V (1)</td>
</tr>
<tr>
<td>Beef meat</td>
<td>2015–2019</td>
<td>127</td>
<td>2 (1.6%)</td>
<td>2</td>
<td>F6 (2)</td>
<td>2 (100%)</td>
<td>Xlb (2)</td>
</tr>
<tr>
<td>Subtotal Meat</td>
<td>2015–2019</td>
<td>468</td>
<td>10 (2.1%)</td>
<td>11</td>
<td>F1 (4), R6 (2), F7 (1), F8 (1), F9 (1), F10 (1)</td>
<td>3 (27.3%)</td>
<td>V (1), Xlb (2)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>710</td>
<td>18 (2.5%)</td>
<td>20</td>
<td>7 (35%)</td>
<td></td>
</tr>
</tbody>
</table>

^a Inside of parenthesis indicates number of isolates.
2.5. Antimicrobial susceptibility testing

Strains were tested for susceptibility to vancomycin, metronidazole, clindamycin, ceftriaxone, erythromycin, ciprofloxacin, and tetracycline (Sigma-Aldrich, St. Louis, MO, US). Minimal inhibitory concentrations (MICs) were measured using the agar dilution method according to guidelines of the Clinical Laboratory Standards Institute [25]. Breakpoints for metronidazole, clindamycin, ceftriaxone, and tetracycline were determined according to the same guidelines. Published data were used to set breakpoints for vancomycin, erythromycin, and ciprofloxacin [21]; C. difficile ATCC700057 was used as the reference strain.

3. Results

3.1. Isolation of C. difficile from retail foods

C. difficile was isolated from eight (3.3%; six taro, one onion, and one burdock) of the 242 vegetables (2016 and 2017), six (6.7%) of the 89 chicken meat (2015 and 2016), one (3.6%) of the 28 chicken liver (2016), one (0.5%) of the 200 pork meat (2019), and two (1.6%) of the 127 beef meat samples (2018 and 2019) (Table 1). All C. difficile-positive vegetables were classified as root vegetables. The rate of C. difficile positivity in root vegetables was 4.7% (8/170). No C. difficile was isolated from the 24 pig liver samples.

From eight vegetable, six chicken meat, one chicken liver, one pork meat, and two beef meat samples, nine, seven, one, one, and two C. difficile isolates were obtained, respectively. When multiple isolates from a single sample exhibited the same PCR ribotype and antimicrobial susceptibility profile, they were considered representative of a single strain. In total, 20 distinct strains were found in this study.

3.2. Characterization of C. difficile isolates from retail foods

Of the 20 C. difficile isolates, 35% (7/20) were positive for either of the toxin genes (Table 1). Among seven toxigenic isolates, three toxigenotypes (V, Xlb, and XIII) were observed based on toxinotyping (Table 1). Toxinotype Xlb was observed in isolates from both vegetables and beef meat.

All 20 strains from retail foods were typable by PCR ribotyping and were resolved into 12 PCR ribotypes (Fig. 1: Ribotype 014, R6, and F1 to F10). The banding patterns of the tested isolates were not matched of those of the control strains except for ribotype 014. Ribotype F1 was the most prevalent among the isolated samples. Ribotype 014 was observed in the isolates from taro. Ribotype R6 was observed in the isolates from burdock, taro, onion, and beef meat. Ribotype R6 was previously observed in C. difficile derived from Japanese cattle feces in our previous study [26].

3.3. Antimicrobial susceptibility

Table 2 summarizes the antimicrobial susceptibility of the 20 isolated strains, which were all susceptible to vancomycin, metronidazole, clindamycin, ceftriaxone, and tetracycline. Resistance to clindamycin, ciprofloxacin, and tetracycline was found in 10 (50%), 18 (90%), and six (30%) of the 20 strains tested, respectively.

4. Discussion

This study showed that 3.3% of vegetables were contaminated with C. difficile in Japan. Previous studies determined that the isolation rates of C. difficile in vegetables were under 10% in each country [7,10,11], similar to that in this study. In contrast, in this study, the isolation rate of C. difficile in meats was 2.1%, whereas such rates were variable (0–42%) in previous reports [7,10,11]. Studies conducted in Europe have persistently reported low prevalence rates in meat samples, in contrast to that in the USA and Canada, where C. difficile is reported at much higher rates [10]. Although it is difficult to compare the isolation rates of C. difficile to those of previous foreign reports, because the isolation condition, year, and portion were different, contamination rates of C. difficile in Japanese retail foods, both vegetables and meats, are not high.
Two reports from Slovenia and Australia showed that isolation rates from vegetables were 18% and 10–30%, respectively [15,16], indicating that root vegetables were more often contaminated with *C. difficile* than other types of vegetables, which is in concordance with the contention that soil is a source of contamination [15,16]. All *C. difficile*-positive vegetable samples in this study were also root vegetables, suggesting that the contamination of vegetables by *C. difficile*, and especially root vegetables, should be monitored in Japan.

Ribotype 014 was observed in one isolate from a vegetable (taro). Ribotype 014 is a prevalent ribotype associated with human clinical cases worldwide including in Japan [18,27,31]. Moreover, this ribotype was reported in calf feces, pork meat, and beef meat in some countries [32]. Furthermore, this ribotype was found to be most prevalent among isolates from vegetables in Slovenia [15]. These results suggest that ribotype 014 could spread among vegetables and humans in Japan.

Ribotype R6 and toxinoctype XIIb were observed in three vegetable- and two beef meat samples in this study. From the results of toxinoctyping, ribotype R6 would be ribotype 288 [33]. This type was also observed in calf feces in our previous study [26]. Although toxinoctype XIIb was previously reported in vegetables and beef meat [34], this type was also frequently reported in cattle and calf feces in foreign countries [35]. These results suggest that this characteristic of *C. difficile* is prevalent in calves and could spread to retail foods.

Some *C. difficile* isolates were also resistant to the tested antimicrobials, which have also been linked to antibiotic-associated diarrhea, caused by *C. difficile* [36]. *C. difficile* antibiotic resistance to treatment is a prominent problem for CDI patients [27]. Studies have demonstrated that the antibiotic resistance profiles of *C. difficile* isolates are quite diverse in different countries [37]. Therefore, more information about the antimicrobial susceptibility profiles of *C. difficile* from several origins is needed.

Considering the present work and other studies conducted on the prevalence of *C. difficile* in retail foods, we speculate that the Japanese population is exposed to low levels of *C. difficile* daily. However, the contamination risk of retail foods is not zero. To prevent the transfer of *C. difficile* to humans from retail foods, it is important to efficiently wash vegetables and heat meats before eating.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgments**

We thank Dr. Haru Kato (National Institute of Infectious Diseases) for providing DNA extracts from some PCR ribotype strains. This work was supported by JSPS KAKENHI (Grant Number 26860441).

**References**


[24] S. Persson, M. Torpdahl, K.E. Olsen, New multiplex PCR method for the detection of *Clostridium difficile* toxin A (tcdA) and toxin B (tcdB) and the


