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High prevalence of *Campylobacter ureolyticus* in stool specimens of children with diarrhea in Japan

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Running Title: *C. ureolyticus* may be a diarrheal pathogen

Keywords: *Campylobacter ureolyticus*, diarrhea, emerging pathogen

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Summary (195/200 words)

Campylobacter ureolyticus has been considered as a potential pathogenic bacterium. In this study, a total 586 stool samples were collected from 0-12 year-old children with diarrhea between November 2013 and April 2015 and examined with microbiological tests in the hospital for diagnosis of common enteric pathogens including C. jejuni and C. coli. Then in our laboratory, these samples were analyzed by 16S rRNA sequence-based Campylobacter genus-specific PCR (C16S PCR) and 283 (48.3%) samples show positive with this PCR assay. Furthermore, C. ureolyticus was screened in these 283 samples by PCR assay, which can detect this species specifically. Surprisingly, C. ureolyticus was detected in 147 out of the 283 C16S PCR-positive diarrheal stool samples (51.9%), which is much higher than the occurrence rate of C. jejuni and C. coli (15.5%), and 96 samples out of 147 were negative for any tested enteric pathogens in hospital, namely C. ureolyticus was detected as a sole pathogen in 96 samples. This finding suggests that C. ureolyticus may be a pathogen associated with diarrhea in children in Japan. To the best of our knowledge, this is the first report showing detection of C. ureolyticus among Japanese children with diarrhea.

Text (1197/1200 words)

Campylobacter jejuni and Campylobacter coli are the most frequently isolated species from human diarrheal patients among 26 Campylobacter species (1). Recently, by using non-selective media and species-specific gene detection methods, other Campylobacter species such as C. concisus and C. upsaliensis, have been increasingly detected and isolated from diarrheal patients (2, 3). Additionally, C. ureolyticus has been suggested to be an emerging pathogen causing gastroenteritis in humans (4, 5). Bullman et al. (4) analyzed 7,194 stool samples from diarrheal patients in Ireland to detect campylobacters and found that the species-specific PCR could detect C. ureolyticus from 83 (22%) out of 373 stool
samples, which were positive for *Campylobacter* spp. As expected, *C. jejuni* (246 samples, 66%) was the most predominant species. Since the study was carried out with patients in limited areas, more epidemiological surveys in different areas are needed to understand whether *C. ureolyticus* is a possible emerging enteric pathogen worldwide. In this study, prevalence of *C. ureolyticus* was examined in stool specimens of children with diarrhea in which *Campylobacter* genus-specific PCR products were obtained in Japan. Simultaneously, all stool samples were subjected to microbiological and molecular diagnosis for the presence of common enteric pathogens.

A total of 586 rectal swabs were collected from 0-12 year-old children with diarrhea who visited Department of Pediatrics, Mizushima Central Hospital, Okayama, Japan between November 2013 and April 2015. Microbiological tests were carried out with culture methods and PCR for enteropathogenic bacteria including *Campylobacter* and *Salmonella*, and immune-chromatography for *Norovirus, Rotavirus* and *Adenovirus* in the hospital for diagnosis of common enteric pathogens. The results of microbiological tests are summarized in Table 1. Simultaneously, the rectal swabs kept in Cary-Blair medium (Becton Dickinson and Company, NJ, USA), were immediately transferred to Osaka Prefecture University at ambient temperature. Then rectal swabs were suspended in 500 µL PBS and DNA template was prepared by boiling method to perform cdt gene-based multiplex PCR for detecting *C. jejuni, C. coli* and *C. fetus* (6), and C16S PCR using *Campylobacter* genus-specific primer set of C16S-F3 (5’-GGAGGATGACACTTTTCG-3’) and C16S-R5 (5’-CGATTACTAGCGATTCCG-3’), which were designed from conserved regions in 16S rRNA genes of 26 *Campylobacter* spp. The PCR mixture contained 0.5 µM of each primer, 1 µL of DNA template, 0.2 mM of dNTP mixture, 1 × rTaq DNA polymerase buffer and 1.0 U of rTaq DNA polymerase (Takara Bio Inc., Shiga, Japan) in a 20 µL reaction volume. PCR was performed in TaKaRa PCR Thermal Cycler (Takara Bio Inc.) or Applied Biosystems
GeneAmp PCR 9700 (Life Technologies Co., Carlsbad, CA). PCR products were analyzed by electrophoresis using 2% PrimeGel™ Agarose LE gel (Takara Bio Inc.) and bands were visualized by UV light after staining with ethidium bromide (1 µg/mL). Images were captured on a Bio-Rad Chemi Doc system (Bio-Rad Laboratories, Inc., Hercules, CA). As shown in Table 1, of 586 samples, 283 (48.3%) were positive for the C16S PCR. Interestingly, the Campylobacter genus-specific PCR amplicon was obtained from not only the 41 samples in which C. jejuni or C. coli was detected at the hospital, but also the 242 samples, which were negative for Campylobacter by using routine culture methods. This finding strongly suggests that the 242 stool samples might contain Campylobacter spp. other than C. jejuni and C. coli.

To identify Campylobacter species in the C16S PCR-positive samples, we first sequenced the C16S PCR amplicons from randomly selected 8 samples in which no enteric pathogen was detected by microbiological tests. Sequencing results indicated the presence of C. hominis which has been proposed as a commensal bacterium in human (7) in 5 samples and C. ureolyticus in 3 samples. Subsequently, we examined the occurrence of C. ureolyticus in the 283 samples that were positive for C16S PCR (Table 1). Corresponding DNA template was subjected to PCR using the C. ureolyticus species-specific PCR primer set developed by Bullman et al. (4). Surprisingly, C. ureolyticus was detected in 147 out of the 283 C16S PCR-positive samples (51.9%), which is much higher than the detection rate of C. jejuni and C. coli (15.5%). It is notable to emphasize that the occurrence rate of C. ureolyticus in stool samples in this study is much higher than that of southern Ireland (22%) (4). To the best of our knowledge, this is the first report showing detection of C. ureolyticus among Japanese children suffering from diarrhea, some of them were detected as a sole pathogen.

In this study, C. ureolyticus was detected in 96 out of 419 samples, which were negative for any common enteric pathogens (Table 1). This finding suggests that C.
ureolyticus may be associated with diarrhea in children in Japan. The reason why C. ureolyticus was not detected in the past could be the culture conditions used in clinical settings of Japan. Isolation of C. ureolyticus needs anaerobic conditions including hydrogen and C. ureolyticus cannot grow on the selective media, which are used for routine isolation of C. jejuni and C. coli. Therefore, C. ureolyticus could be easily overlooked when attempted to isolate Campylobacters from diarrheal patient. Indeed, when we attempted to isolate C. ureolyticus by using blood agar with nalidixic acid, vancomycin and amphotericin B under anaerobic condition with hydrogen (80% N₂, 10% CO₂, 10% H₂), we could successfully isolate C. ureolyticus from diarrheal stool samples of children (data not shown).

C. ureolyticus was reclassified from Bacteroides ureolyticus in 2010. The pathogenicity of this species remains unclear. In 2012, Burgos-Portugal et al. showed that C. ureolyticus has the ability to attach and translocate to human intestinal epithelial cell lines (8). Thereafter, analysis of whole genome sequence of C. ureolyticus was carried out and at least 106 potential virulence-related genes were found in this species (9). Therefore, C. ureolyticus has been considered to be potentially pathogenic. Mukhopadhyya et al. reported that this species was significantly associated with ulcerative colitis in adults (10). In this study, we show that in Japanese children with diarrhea, detection rate of C. ureolyticus was much higher than that from a recent survey in southern Ireland (4). Additionally, we observed that C. ureolyticus was frequently detected in the samples that were negative for any common enteric pathogens. These findings suggest C. ureolyticus may be associated with diarrhea in children in Japan. However, in this study, 51 C. ureolyticus were detected from 167 samples in which other enteric pathogens were also detected and the prevalence of this species was analyzed in only children with diarrhea. Therefore, a case-control study is required to understand whether C. ureolyticus is associated with diarrhea in children in Japan.
In conclusion, *C. ureolyticus* may be associated with diarrhea in children in Japan. Further surveillance of enteric pathogen, particularly *C. ureolyticus*, in diarrheal children is required to understand the importance of *C. ureolyticus* as an enteric pathogen by using both culture and genetic methods.

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**Conflict of interest:** None to declare.

**References**


Table 1: Isolation and detection of enteric pathogens, *Campylobacter* spp. and *C. ureolyticus* in children with diarrhea

<table>
<thead>
<tr>
<th>Pathogens detected*¹</th>
<th>Number of patient</th>
<th>C16S PCR*²</th>
<th><em>C. ureolyticus</em>³</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni / C. coli</em></td>
<td>41</td>
<td>40</td>
<td>18</td>
</tr>
<tr>
<td><em>C. jejuni / C. coli and Norovirus</em></td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>22</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td><em>Norovirus</em></td>
<td>41</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td><em>Rotavirus</em></td>
<td>39</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td><em>Adenovirus</em></td>
<td>10</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Others*⁴</td>
<td>11</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Subtotal</strong>*⁵</td>
<td>167</td>
<td>97</td>
<td>51</td>
</tr>
<tr>
<td><strong>ND</strong>*⁶</td>
<td>419</td>
<td>186</td>
<td>96</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>586</td>
<td>283</td>
<td>147</td>
</tr>
</tbody>
</table>

*¹Pathogens were detected and isolated at Mizushima Central Hospital. *²No. of positive specimens by the C16S PCR. *³No. of positive specimens by the *C. ureolyticus*-specific PCR. *⁴Others including *Influenzavirus, Yersinia* spp., *Klebsiella oxytoca*, both *Aeromonas* spp. and *Norovirus*, and *Streptococcus* spp., *⁵No. of total samples which other enteric pathogens were detected in hospital. *⁶ND: not detected.