Jpn. J. Infect. Dis., 70, 455–457, 2017

Short Communication

High Prevalence of *Campylobacter ureolyticus* in Stool Specimens of Children with Diarrhea in Japan

Noritoshi Hatanaka[1†], Akinori Shimizu[1†], Srinuan Somroop[1], Yiming Li[1,2], Masahiro Asakura[1], Akira Nagita[3], Sharda Prasad Awasthi[1], Atsushi Hinenoya[1], and Shinji Yamasaki[1*]

[1]Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531; [3]Department of Pediatrics, Mizushima Central Hospital, Okayama 712-8064, Japan; and [2]School of Light Industry and Food Science, South China University of Technology, Guangzhou, China

SUMMARY: *Campylobacter ureolyticus* has been considered as a potentially pathogenic bacterium. In this study, a total of 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 the 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); 283 (48.3%) samples showed positive results 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 of the 283 C16S PCR-positive diarrheal stool samples (51.9%), which is much higher than the prevalence of *C. jejuni* and *C. coli* (15.5%), and 96 samples out of 147 were negative for any of the other enteric pathogens tested in the hospital; namely, *C. ureolyticus* was detected as a single 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 in which *C. ureolyticus* was detected among Japanese children with diarrhea.

Among 26 *Campylobacter* species (spp.) *C. jejuni* and *C. coli* are the most frequently isolated species from human diarrheal patients (1). Recently, by using non-selective media and species-specific gene detection methods, other *Campylobacter* spp. 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 *Campylobacter* 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 a limited area, more epidemiological surveys in different areas are needed to understand whether *C. ureolyticus* is a possible emerging enteric pathogen worldwide. In this study, the 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.

Received September 26, 2016. Accepted December 13, 2016. J-STAGE Advance Publication February 28, 2017.
DOI: 10.7883/yoken.JJID.2016.428

*Corresponding author: Mailing address: Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58, Rinkuourai-kita, Izumisano, Osaka 598-8531, Japan. Tel: +81-72-463-5653, E-mail: shinji@vet.osakafu-u.ac.jp

† These authors contributed equally to this work.

A total of 586 rectal swabs were collected from 0–12-year-old children with diarrhea who visited the Department of Pediatrics, Mizushima Central Hospital, Okayama, Japan between November 2013 and April 2015. Microbiological tests were carried out with culture methods and PCR was performed for enteropathogenic bacteria including *Campylobacter* and *Salmonella*, and immunochromatography was performed for Norovirus, Rotavirus, and Adenovirus in the hospital for the diagnosis of common enteric pathogens. The results of microbiological tests are summarized in Table 1.

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

Pathogens detected[1)]	Number of patient	C16S PCR[2)]	*C. ureolyticus*[3)]
C. jejuni/*C. coli*	41	40	18
C. jejuni/*C. coli* and Norovirus	3	3	1
Salmonella spp.	22	9	5
Norovirus	41	14	7
Rotavirus	39	22	13
Adenovirus	10	7	5
Others[4)]	11	2	2
Subtotal[5)]	167	97	51
ND[6)]	419	186	96
Total	586	283	147

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

Simultaneously, the rectal swabs kept in Cary-Blair medium (Becton Dickinson and Company, Franklin Lakes, NJ, USA), were immediately transferred to Osaka Prefecture University at an ambient temperature. Then rectal swabs were suspended in 500 μL phosphate-buffered saline and a DNA template was prepared by the boiling method to perform *cdt* gene-based multiplex PCR for detecting *C. jejuni, C. coli,* and *C. fetus* (6), and C16S PCR using a *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 the DNA template, 0.2 mM of dNTP mixture, r*Taq* DNA polymerase buffer, and 1.0 U of r*Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) in a 20 μL reaction volume. PCR was performed in the TaKaRa PCR Thermal Cycler (Takara Bio Inc.) or Applied Biosystems GeneAmp PCR 9700 (Life Technologies Co., Carlsbad, CA, USA). 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, USA). 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* 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* spp. in the C16S PCR-positive samples, we first sequenced the C16S PCR amplicons from 8 randomly selected 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 humans (7), in 5 samples and *C. ureolyticus* in 3 samples. Subsequently, we examined the prevalence of *C. ureolyticus* in the 283 samples that were positive for C16S PCR (Table 1). The corresponding DNA template was subjected to PCR using the *C. ureolyticus* spp.-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 prevalence rate of *C. ureolyticus* in stool samples in this study is much higher than that in southern Ireland (22%) (4). To the best of our knowledge, this is the first report of a study wherein *C. ureolyticus* was detected among Japanese children with diarrhea, and in some of whom, it was detected as a single 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* requires 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 attempting to isolate *Campylobacter* from samples from patients with diarrhea. 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% N_2, 10% CO_2, 10% H_2), 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 cells (8). Thereafter, analysis of the 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. Mukhopadhya et al. reported that this species was significantly associated with ulcerative colitis in adults (10). In this study, we showed that in Japanese children with diarrhea, the detection rate of *C. ureolyticus* was much higher than that reported by 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 using routine methods. However, in this study, *C. ureolyticus* was isolated in 51 out of 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 pathogens, particularly *C. ureolyticus*, in children with diarrhea is required to understand its importance as an enteric pathogen by using both culture and genetic methods.

Acknowledgments We thank Dr. R. K. Bhadra, CSIR-Indian Institute of Chemical Biology, Kolkata, India and Dr. J. Lin, Department of Animal Science, The University of Tennessee, Knoxville TN, USA for critically reading the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the JSPS and for Scientific Research of US-Japan Cooperative Medical Science Program from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None to declare.

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