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Analysis of *Clostridium* cluster XI bacteria in human feces

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Six species and one group of *Clostridium* cluster XI, *Clostridium sordellii, Clostridium bifermentans, Clostridium difficile, Clostridium hiranonis, Intestinibacter bartlettii,* and *Romboutsia lituseburensis* and the *Terrisporobacter glycolicus* group, respectively, in human feces collected from 18 healthy adults were analyzed with real-time PCR. Although individual differences were recognized, the predominant colonization of *C. sordellii* and *I. bartlettii* in the human large intestine was identified.

Key words: *Clostridium* cluster XI, real-time PCR, human feces

Clostridia are among the predominant bacteria in the human large intestine. This genus is divided into 19 clusters according to phylogenetic analysis of the 16S rRNA gene [1]. Based on this analysis, many of the bacteria phylogenetically related to *Clostridium* in the human large intestine belong to *Clostridium* clusters I, IV, XI, XIVa, and XVI. It was suggested that a high intake of carbohydrates, fat, and protein was associated with increasing amounts of *Clostridium* cluster XI bacteria in the feces of patients with type 2 diabetes mellitus [2]. Furthermore, it was suggested that the stimulation of *Clostridium* cluster XI in the feces of mice resulting from feeding of a high-fat diet was associated with development of hepatocellular carcinoma due to the increase of deoxycholic acid produced by the 7α-dehydroxylation enzyme derived from *Clostridium* cluster XI bacteria [3]. Thus, *Clostridium* cluster XI should be one of the important harmful bacteria in the large intestine. It is, however, unclear which *Clostridium* cluster XI bacteria induce the development of hepatocellular carcinoma.

Within *Clostridium* cluster XI, *Clostridium difficile,* which was reclassified as *Clostridioides difficile* [4]; *Clostridium sordellii*, and *Clostridium bifermentans* are well-known bacterial species in the human large intestine. *C. difficile* is known to cause antibiotic-associated diarrhea and pseudomembranous colitis [5]. *C. sordellii* and *C. bifermentans* have 7α-dehydroxylating activity, which is associated with carcinogenesis [6]. In addition, *Clostridium hiranonis,* which also has 7α-dehydroxylating activity, was identified from human feces [7]. Secondary bile acids produced by the 7α-dehydroxylation enzyme are suspected of being carcinogens in colorectal cancer. Therefore, *C. sordellii*, *C. bifermentans*, and *C. hiranonis* have been recognized as harmful bacteria. Within *Clostridium* cluster XI, *Clostridium bartlettii*, *Clostridium glycolicum*, and *Clostridium lituseburense*, which were reclassified as *Intestinibacter bartlettii, Terrisporobacter glycolicus,* and *Romboutsia lituseburensis*, respectively [8], were also identified from human feces [9, 10].

It is important to identify which species within *Clostridium* cluster XI inhabit the human large intestine. In this study, we analyzed *C. difficile*, *C. sordellii*, *C. bifermentans*, *C. hiranonis*, *R. lituseburensis*, *I. bartlettii*, and *T. glycolicus* using real-time PCR to reveal the composition of *Clostridium* cluster XI bacteria in human feces.

Eighteen fecal samples collected from 16 female and 2 male healthy volunteers age 21–22 were analyzed. The volunteers consumed nonspecific Japanese diets and took no antibiotics for one month prior to fecal collection. This study was approved by the Nippon Veterinary Life Science University Ethics Committee and was performed in accordance with the Helsinki Declaration as updated in Brazil in 2013. The details of this study were explained to all volunteers. An informed consent agreement was obtained from all volunteers before the experiment. Bacterial DNA was extracted from 0.1 g of feces in accordance with the methods of Godon *et al.* [11].

Primers for *C. hiranonis*, *R. lituseburensis*, *I. bartlettii*, and *T. glycolicus* were designed. The specificities of these primers were verified with the BLAST provided by the DNA Data of Bank of Japan and the sequencing of PCR products amplified by these primers. For direct sequencing of PCR products, PCR was done with the following conditions: 25 µl of GoTaq (Promega, Tokyo, Japan), 400 µmol/l of each primer, and 2 µl of extracted bacterial DNA in a total volume of 50 µl. Three fecal bacterial DNA were randomly selected from eighteen samples for one primer set, except for *C. hiranois* primers. For *C. hiranois* primers, one fecal bacterial DNA was used for PCR, because PCR products were obtained from only one sample. The thermal program consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of at 95°C for 30 sec, primer annealing at optimum temperature

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Table 1. Primers used in the present study

Target	Primer	Sequence (5' – 3')	Annealing (˚C) / elongation (sec)	Reference
Clostridium cluster XI	ClostXI-F	ACGGTACTTGAGGAGGA	53 / 15	[12]
	ClostXI-R	GAGCCGTAGCCTTTCACT		
C. sordellii	CLSOR-F	TCGAGCGACCTTCGG	54 / 60	[13]
	CLSOR-R	CACCACCTGTCACCAT		
C. bifermentans	CLBIF-F	CAAGTCGAGCGATCTCT	59 / 30	[13]
	CLBIF-R	CCTGCACTCAAGTTCTCT		
C. difficile	CLDIF-F	CTTGAATATCAAAGGTGAGCCA	54 / 60	[13]
	CLDIF-R	CTACAATCCGAACTGAGAGTA		
C. hiranonis	Chira-F	GTAAGCTCCTGATACTGTCT	50 / 25	This study
	Chira-R	GGGAAAGAGGAGATTAGTCC		
I. bartlettii	Cbart-F	GTAAGCTCTTGAAACTGGAG	59 / 25	This study
	Cbart-R	GAAAGATGCGATTAGGCATC		
T. glycolicus	Cgly-F2	AAGCTCCGGCGGTATGA	54 / 35	This study
	Cgly-R3	CTCTCCTGCACTCAAGTCTC		
R. lituseburensis	Clitu-F	TGACATCCTTTTGACCTCTC	54 / 35	This study
	Clitu-R	GCCTCACGACTTGGCTG		

Table 2. Identification of PCR products derived from PCR using primers designed in this study

Primers	Closest relative [GenBank accession number]	Identity (%)
Chira-F, Chira-R	*C. hiranonis* [JF693906]	99
Clitu-F, Clitu-R	*R. lituseburensis* [MF988703]	100
	R. lituseburensis [MF988703]	100
	R. lituseburensis [MF988703]	100
Cgly-F2, Cgly-R3	*C. glycolicum*[1] [X76750] / *C. mayombei*[2] [FR733682]	99
	C. glycolicum [X76750] / *C. mayombei* [FR733682]	99
	C. glycolicum [X76750] / *C. mayombei* [FR733682]	99
Cbart-F, Cbart-R	*C. bartlettii*[3] [AY438672]	99
	C. bartlettii [AY438672]	100
	C. bartlettii [AY438672]	100

[1] *C. glycolicum* is currently classified as *T. glycolicus.*
[2] *C. mayombei* is currently classified as *T. mayombei.*
[3] *C. bartlettii* is currently classified as *I. bartlettii.*

for 30 sec and at 72°C for optimum elongation length, and final elongation at 72°C for 5 min. The optimum temperature and elongation length of each primer set are given in Table 1. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and subjected to sequencing (Eurofins Genomics, Tokyo, Japan). The obtained sequences were subjected to BLAST search. As shown in Table 2, the sequences of PCR products used with primers for *C. hiranonis*, *R. lituseburensis*, and *I. bartlettii* were related to *C. hiranonis* [JF693906], *R. lituseburensis* [MF988703], and *Clostridium bartlettii* [AY438672], respectively, with 99% or 100% similarities. These results indicated that these primers were effective for the detection of their target bacteria. The sequences of PCR products used with primers for *T. glycolicus* were related to *C. glycolicum* [X7650] and *Clostridium mayombei* [FR733682], which was reclassified as *Terrisporobacter mayombei* with 99% similarity. Therefore, the target of primers for *T. glycolicus* was evaluated as the *T. glycolicus* group in this study. However, there are no reports to our knowledge indicating that *T. mayombei* has been found in human feces. We considered that *T. glycolicus* might be effectively detected in human feces by PCR using the primer for *T. glycolicus.*

Using these and previously reported primers [12, 13], the 16S rRNA genes of *C. difficile*, *C. sordellii*, *C. bifermentans*, *C. hiranonis*, *R. lituseburensis*, *I. bartlettii*, *T. glycolicus* group, and *Clostridium* cluster XI were quantified via real-time PCR. Real-time PCR was performed using a MyiQ real-time PCR system (Bio-Rad, Tokyo, Japan). The reaction mixture (20 µl) contained 10 µl of the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.5 µl of fecal DNA, and 400 µmol/l of each primer. The primers used in this study are listed in Table 1. The thermal program consisted of initial denaturation at 95°C for 3 min, followed by 40 cycles of at 95°C for 10 sec,

Table 3. Detection of the 16S rRNA gene of *Clostridium* cluster XI bacteria in human feces

Bacteria	Number of 16S rRNA genes (log copy number of 16S rRNA gene/g of feces)	Number of detective subjects (%)
Clostridium cluster XI	9.43 ± 0.42	18 (100)
C. sordellii	8.84 ± 0.40	18 (100)
I. bartlettii	8.17 ± 0.86	18 (100)
C. bifermentans	6.68 ± 0.44	14 (77.8)
T. glycolicus group	6.42 ± 0.78	8 (44.4)
R. lituseburensis	6.14 ± 0.87	6 (33.3)
C. hiranonis	9.56	1 (5.6)

16S rRNA gene values are the means ± SD.

primer annealing at the optimum temperature for 30 sec and at 72°C for the optimum elongation length, and final elongation at 72°C for 5 min. The optimum temperature and elongation length of each primer set are given in Table 1. The fluorescent products were detected in the last step of each cycle. A melting-curve analysis of the product was performed after completion of the amplifications to determine the specificity of the PCR. A plasmid containing a partial sequence of the 16S rRNA gene identical to the targeted bacteria was constructed in our laboratory and used as a standard DNA for the real-time PCR. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Clostridium cluster XI was detected in all subjects, with the $\log_{10}$ copy number of rRNA gene/g of feces ranging from 8.57 to 10.02. There was a large individual difference in the number of *Clostridium* cluster XI bacteria colonizing in the human large intestine. Although the number of *Clostridium* cluster XI in the human large intestine may be less than the number of predominant bacteria, such as *Bifidobacterium*, *Bacteroides*, and *Clostridium* cluster XIVa [9], in the human large intestine, it was considered that *Clostridium* cluster XI should be one of the bacterial group constructing the human large intestinal microbiota. Since *Clostridium* cluster XI might be undesirable for host health as mentioned above, it is more important to clarify which species in *Clostridium* cluster XI are present in the human large intestine.

The number of species among seven targeted *Clostridium* cluster XI bacteria detected in a subject ranged from 3 to 5. Four species of *Clostridium* cluster XI were detected in a subject on average. *C. sordellii* was detected in all subjects and showed the highest number of 16S rRNA genes (Table 3). *I. bartlettii* was also detected in all subjects. The detection rates of *C. bifermentans*, *C. hiranonis*, *R. lituseburensis*, and *T. glycolicus* group were 77.8, 5.6, 33.3, and 44.4%, respectively. The copy numbers of the 16S rRNA gense of *C. bifermentans*, *R. lituseburensis*, and *T. glycolicus* were 100 to 1,000 times lower than those of *C. sordellii* and *I. bartlettii.* Although the copy number of the 16S rRNA gene of *C. hiranonis* was higher than those of *C. sordellii* and *I. bartlettii, C. hiranonis* was detected in only one subject. These results suggested that *C. sordellii* and *I. bartlettii*, particularly *C. sordellii*, might be predominant *Clostridium* cluster XI bacterial species colonizing the human large intestine. *C. sordellii* could be considered one of the important harmful bacteria in human large intestinal microbiota associated with carcinogenesis, since this bacterium has 7α-dehydroxylating activity, as mentioned above.

On the other hand, *C. difficile* was not detected in all subjects. Low colonization of *C. difficile* in healthy adults has been reported [5, 14, 15]. Moreover, in most cases, the number of *C. difficile* in healthy adult feces was lower than 10^4 cfu/g [14, 16]. Thus, it has been shown that *C. difficile* is poorly colonized in the large intestine of most healthy young adults [5, 15]. To detect target bacteria via PCR, more than 10^5 cells/g of feces is required due to the sensitivity of the PCR [17]. Therefore, in most healthy young adults, it might be difficult to detect *C. difficile* in feces using real-time PCR . In this study, the number of *C. difficile* in the feces of all subjects may have been below the detection limit (10^4 cfu/g of feces). This might be also true for the case in which *C. bifermentans*, *C. hiranonis*, *R. lituseburensis*, and *T. glycolicus* group were not detected. In particular, it was suggested that detection of *C. hiranonis* by PCR was difficult due to the population of *C. hiranonis* being small even if the bacterium colonized in the large intestine [18]. Kubota *et al.* reported that TaqMan-based qPCR can detect *C. difficile* at 10^3 cfu/g of feces [16]. Small numbers of bacteria were effectively analyzed via RNA-targeted reverse transcription-PCR [17]. To detect these minor bacteria in feces, further studies using these methods are necessary.

The copy number of the 16S rRNA gene of *Clostridium* cluster XI was approximately equal to the sum of those of each analyzed species, except in 2 subjects. This suggested that *C. sordellii*, *I. bartlettii*, *C. bifermentans*, *C. hiranonis*, *R. lituseburensis*, and *T. glycolicus* group might contribute to the composition of *Clostridium* cluster XI microbiota in the large intestine of young adults. However, in the abovementioned 2 subjects, the copy number of the 16S rRNA gene of *Clostridium* cluster XI was more than 10 times larger than the sum of those of each analyzed species. This suggested that the other bacterial species belonging to *Clostridium* cluster XI might colonize in the large intestine. The colonization of *Clostridium ghonii*, *Clostridium irregulare*, and *Eubacterium tenue*, which belong to *Clostridium* cluster XI, in human

feces was previously reported [9]. These bacteria may also be members of the indigenous bacteria comprising *Clostridium* cluster XI microbiota in the human large intestine.

Although not all species of *Clostridium* cluster XI bacteria were analyzed, we could analyze the composition of *Clostridium* cluster XI in the human large intestine in detail using real-time PCR. In this study, the individual differences in the numbers and kinds of bacterial species of *Clostridium* cluster XI were recognized. In addition, the predominant colonization of *C. sordellii* and *I. bartlettii* in the large intestine of young adults was identified. Analyses of each bacterial species of *Clostridium* cluster XI in the human large intestine as in this experiment could provide further detail and effectively clarify the relationship between *Clostridium* cluster XI bacteria and host health.

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