Original Articles

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Detection of *Arcobacter* Species in Human Stool Samples by Culture and Real-time PCR

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***Objective*:** The clinical significance of *Arcobacter* species has not been established due to a lack of suitable detection methods.
***Material and Methods*:** A total of 1,650 stool samples submitted to the Clinical Laboratory of Heidelberg University Hospital were inoculated onto agar plates selective for *Campylobacter* species isolation and incubated at 37℃.
***Results*:** Four (0.24%) of the samples were positive for *Arcobacter butzleri* isolates. Genus-specific primers for real-time PCR were designed to identify *Arcobacter* species. Of the 1,650 stool samples tested, twelve (0.73%), including the four culture-positive samples, were positive for *Arcobacter* species by real-time PCR. The sensitivity of real-time PCR was 10^4 CFU g^{-1} stool, 50 CFU $reaction^{-1}$ using a stool sample.
***Conclusions*:** Although the sensitivity of real-time PCR was relatively low compared with other PCR methods, the present method detected a broad range of *Arcobacter* species. The combination of the stool culture using agar selective for *Campylobacter* species and real-time PCR for *Arcobacter* species may be clinically useful for the diagnosis and epidemiology of *Arcobacter* species infections.

***Key words*:** *Arcobacter* species, human, stool culture, real-time PCR

Introduction

The genus *Arcobacter* belongs to the class *Epsilonproteobacteria*, which includes two families, family *Helicobacteraceae* with *Helicobacter pylori*, which is famous as a cause of gastric cancer, and family *Campylobacteraceae*. The family *Campylobacteraceae* includes two genera, the genus *Campylobacter* and the genus *Arcobacter*, and the genus *Arcobacter* is currently containing at least 28 species[1]. *Arcobacter* species are present in various animals, including livestock and environments in some regions and countries. Although infection caused by *Arcobacter* species is rare, three species, *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*, have been associated with enteritis and occasional bacteremia in human[2].

The morphological and biochemical properties of *Arcobacter* species are similar to those of *Campylobacter* species, as are the clinical features of patients infected with these pathogens are similar to those of *Campylobacter* species[3]. However, the bacteriological

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properties of *Arcobacter* species differ from those of *Campylobacter* species. For example, *Arcobacter* species can grow in the presence of oxygen (aerotolerance) and at lower temperatures (15 to 37℃) than *Campylobacter* species (35-42℃)[4) 5)].

Symptoms of *A. butzleri* infection include abdominal pain, diarrhea, nausea, vomiting and fever[6)]. Moreover, *A. butzleri* infection is more frequently associated with persistent and watery diarrhea, and less frequently associated with bloody diarrhea, than *Campylobacter jejuni* infection[6)]. *Arcobacter* species infection has become increasingly important as an emerging gastrointestinal infection[3)]. *Arcobacter* species infection in patients with other underlying diseases, including diabetes mellitus[7)], liver cirrhosis[8)], cancer and chronic renal failure[9)], may become severe enough to require hospitalization and medication. An outbreak of *A. butzleri* infection was reported to affect 10 children in a nursery and elementary school in Italy[10)]. Moreover, bacteremia caused by *A. butzleri* in a neonate was regarded as vertically transmitted[11)].

The prevalence of *Arcobacter* species in stool of patients with enteritis have been reported to range from 0.1% to 1.5% using culture methods, and from 0.4 to 57.0% using PCR methods[3) 4) 5) 12)]. However, the rates of detection of *Arcobacter* species in clinical laboratories are likely underestimated because there are no standardized protocols for the detection of these species[3)] and because it is difficult to identify *Arcobacter* species using routine methods for bacterial identification[13)]. This study describes a method to detect clinical isolates of *Arcobacter* species in stool samples, involving culture of stool samples and genus specific real-time PCR.

Materials and Methods

1. Patient stool samples for culture and real-time PCR

A total of 10,133 stool samples were submitted to the microbiology laboratory of Heidelberg University Hospital in Germany from December 2015 to March 2017. These samples were maintained at room temperature and tested within 24 hours by culturing and isolation of bacterial pathogens. Following culture inoculation, the remaining samples were stored at -20℃. Of all the stored samples, 1,650 samples were tested for real-time PCR. All samples tested for real-time PCR were obtained from patients in the internal medicine department of the University Hospital or the affiliated local primary care hospitals. Multiple samples obtained from a single patient were tested individually.

2. Identification of *Arcobacter* species from stool culture

Stool samples were cultured by routine methods to isolate species of the genera *Aeromonas*, *Campylobacter*, *Salmonella*, *Shigella*, and *Yersinia*. Briefly, stool samples were streaked on Columbia agar containing 5% sheep blood, on xylose-lysine-deoxycholate (XLD) agar (Becton Dickinson, Franklin Lakes, NJ, USA), on *Campylobacter* selective agar (Campylosel agar®; bioMerieux) and on cefsulodin-irgasan-novobiocin (CIN) agar (Becton Dickinson) using PREVI® Isola automated streaker (bioMerieux). Columbia and XLD agar plates were incubated under aerobic conditions at 37℃ for 24 hours; *Campylobacter* selective agar plates were incubated under microaerobic conditions at 37℃ for 48 hours; and CIN agar plates were incubated under aerobic conditions at 30℃ for 24 hours. Stool samples were also transferred to Selenite broth (Becton Dickinson) for enrichment; after incubation under aerobic conditions at 37℃ for 24 hours, the broth was streaked on XLD agar plates and incubated under aerobic conditions at 37℃ for 24 hours. Colonies on the culture plates were identified by MALDI Biotyper® (Bruker Daltonik, Billerica, MA, USA) which is based on proteomic fingerprinting using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) technique for colony identification, because mass spectrometry is more reliable in identifying *Arcobacter* species[14)]. We identified colonies with the score 2.0 or greater as *Arcobacter* species.

3. Identification of *Arcobacter* species by real-time PCR

1) Primer and probe design

The 23S rRNA sequences of 16 *Arcobacter* species were obtained from National Center for Biotechnology Information (NCBI) Taxonomy Database in January 2016[1)]. The sequences were aligned with BioEdit v7.0.5[15)] and the primers and a probe were designed using Primer3[16)] to amplify regions of *A. butzleri* 23S rRNA. Specificity of the

Table-1 Primer and probe sequences based on the *Arcobacter butzleri* 23S rRNA for detection of *Arcobacter* species

Primer/Probe	Sequence (5'-3')	Position	Size	Tm	GC content
Forward primer	GTCGTGCCAAGAGCCA	1593-1612	19 bp	58.0℃	52.60%
Reverse primer	TTCGCTTGCGCTGACAT	1893-1910	17 bp	52.0℃	52.90%
Probe	CACCGGGCAGGCGTCACACCTTATAC	1849-1875	26 bp	59.2℃	61.50%

Strain and primer position in 23S rRNA (forward, reverse)

Arcobacter species
Arcobacter butzleri RM4018 (1588-1606, 1887-1903) 5'--ACTGTCGTGCCAAGAAAAGCCACTA----------ATGATGTCAGCGCAAGCGAAGCA--3'
Arcobacter skirrowii L403 629 (1588-1606, 1887-1903) GTCGTGCCAAGAAAAGCCA ATGTCAGCGCAAGCGAA

Helicobacter species
Helicobacter pylori 26695 (1675-1693, 2413-2429) GTCGTGCCAAGAAAAGTCT AGGCCAGGACACGAGAA
Helicobacter cinaedi CCUG 18818 (ATCC BAA-847) (1588-1606, 391-407) GTCGTGCCAAGAAAAGCCT AGGCCAGGACACGTGAA

Campylobacter species
Campylobacter jejuni NCTC 11168 (ATCC 700819) (1579-1597, 1877-1893) GTCGAGCCAAGAAAAGTTT GGGTTAGCATTAGCGAA
Campylobacter coli OR12 (1738-1756, 2036-2052) GTCGAGCCAAGAAAAGTTT GGGTTAGCATTAGCGAA

Figure-1 Specificity of the primers for 23S rRNA of *Arcobacter* species
The sequences of 23S rRNA of the representative strains of the class *Epsilonproteobacteria*, of which whole-genome sequences were available in NCBI genome site, were collected and aligned with the primers to detect *Arcobacter* species in this study. The primers developed in this study were specific for *Arcobacter* species but could not detect *Helicobacter* species and *Campylobacter* species.

primers was checked using the primer-blast tools from NCBI. The primers were complementary to nucleotides 1593-1612 (forward primer) and 1893-1910 (reverse primer) and the probe to nucleotides 1849-1875, with the reverse primer and probe binding to the reverse strand (Table-1). The size of PCR product was 317 base pairs. The specificity of the primers for *Arcobacter* species 23S rRNA was shown in Figure-1, by alignment with the sequences of representative strains of *Helicobacter pylori, Helicobacter coli, Campylobacter jejuni* and *Campylobacter coli*. Alignment was performed using MEGA7[17].

2) Preparation of pooled stool samples

Mixtures of stool samples were prepared from 10 individual samples. Briefly, a pea-sized amount or 250 μ*l* of each sample was transferred to a sterile tube containing 5 m*l* nuclease-free water. Each of the 165 pooled stool samples was homogenized and filtrated with a 5 μm syringe filter. Filtrates were collected and centrifuged at 3,000 rpm for 10 minutes. The supernatants were discarded and DNA was extracted from the re-suspended pellets.

3) DNA extraction

DNA was extracted from the pooled samples using QIAamp Fast DNA Stool Mini Kit® (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. If the pooled sample was positive for *Arcobacter* species by real-time PCR, DNA was extracted separately from each of the 10 individual stool samples that comprised the mixture.

4) Real-time PCR

Real-time PCR was performed using a Smartcycler® (Cepheid, Sunnyvale, CA, USA). Each 25 μ*l* reaction mixture consisted of 12.5 μ*l* of 1 × Takyon Master Mix® (Eurogentec, Seraing, Belgium), 6.63 μ*l* of nuclease free water, 0.25 μ*l* of 500 nM forward primer, 0.25 μ*l* of 500 nM reverse primer, 0.38 μ*l* of 150 nM probe and 5 μ*l* of template DNA. Five μ*l* of nuclease free water was used as a negative control and 5 μ*l* of 1.0×10^{-2} ng/m*l* *A. butzleri* DSM_8739 (type strain) DNA solution was used as a positive control. The amplification protocol consisted of an initial denaturation at 95℃ for 3 minutes, followed by 40 cycles of denaturation at 95℃ for 45 seconds, annealing at 61℃ for 30 seconds and extension at 72℃ for 30 seconds. PCR for DNA extracted from the mixture of 10 samples and following PCR using DNA extracted separately from each of the 10 individual stool samples used the same PCR protocol.

5) Sequencing of the PCR products

The PCR product was sent out to GATC Biotech (Konstanz, Germany) and sequenced with the same primer sets for PCR, by Sanger sequencing method. Each sequence was submitted to leBIBI-QBPP r_procaryota_LSU-rDNA-23S_stromgemt database[18] to confirm that the sequence of the PCR product was that of an *Arcobacter* species and to identify each species of *Arcobacter*.

6) Stool culture from frozen PCR-positive stool samples

Stool samples positive for *Arcobacter* species by real-time PCR and stored at -20℃ were retrieved and cultured. Briefly, 1 gram of each stool sample was transferred to 9 m*l* *Arcobacter* enrichment broth, containing 24 g/*l* *Arcobacter* broth (Thermo Scientific, Waltham, MA, USA) and selective supplement (100 mg l^{-1} 5-fluorouracil, 10mg l^{-1} amphotericin B, 16 mg l^{-1} cefoperazone, 32 mg l^{-1} novobiocine and 64 mg l^{-1} trimethoprim)[19]. After incubation under microaerobic conditions at 37℃ for 48 hours, the broth was streaked onto *Campylobacter* selective agar (Campylosel agar®; bioMerieux) and incubated under microaerobic conditions at 37℃ for 120 hours. Colonies were identified by MALDI Biotyper®(Bruker Daltonik).

7) Sensitivity and specificity of real-time PCR assay for *Arcobacter* species (Figure-2)

Sensitivity of real-time PCR assay was assessed using a stool sample negative for *A. butzleri* by real-time PCR (background matrix), spiked with *A. butzleri* DSM_8739. Sensitivity was also assessed using a PBS spiked with *A. butzleri* DSM_8739. Briefly, 200 mg aliquots of stool (background matrix) or 200 μ*l* aliquiots of PBS were seeded with 10-fold serial dilutions of *A. butzleri* DSM_8739 solution at concentrations ranging from 1.0×10^6 CFU ml^{-1} to 10^1 CFU ml^{-1} (or g^{-1} of stool). The negative control consisted of unseeded stool (background matrix) or PBS. DNA was extracted and real-time PCR was performed as above to determine sensitivity of the real-time PCR assay.

Sensitivity of the primers was assessed using 10-fold serial dilution of PCR product solution at concentrations ranging from 1.37×10^5 copy μl^{-1} to 1.37×10^{-1} copy μl^{-1}.

Specificity of the primers was assessed using enteropathogenic bacteria including *A. butzleri*. Briefly, DNA of *Aeromonas hydrophila*, *Arcobacter butzleri*_DSM8739, *Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli* O-157, *Salmonella typhimurium* and *Shigella sonnei* was extracted respectively and PCR was performed using

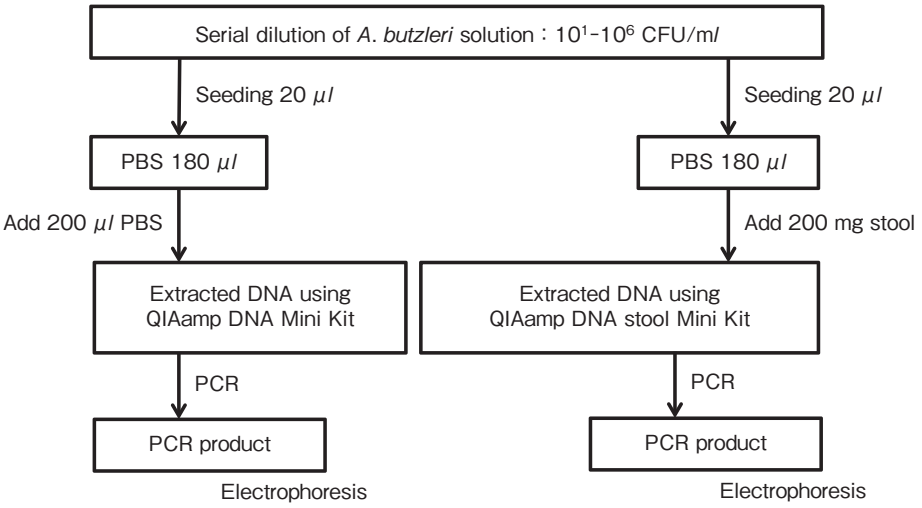

Figure-2 Assessment of real-time PCR assay sensitivity with or without stool
Assessment of real-time PCR assay sensitivity with or without stool. Two hundred milligram aliquots of stool (background matrix) or 200 μ*l* aliquiots of PBS were seeded with 10-fold serial dilutions of *Arcobcter butzleri* DSM_8739 solution at concentrations ranging from 1.0×10^6 CFU ml^{-1} to 10^1 CFU ml^{-1} (or g^{-1} of stool). DNA was extracted and real-time PCR was performed to detect *Arcobcter butzleri* to know sensitivity of the assay with or without stool.

Table-2 Primer sequences based on the housekeeping gene of the 16S rRNA for detection of all bacteria universally

Primer/Probe	Sequence (5'-3')	Size	Tm	GC content
27 Forward primer	AGAGTTTGATCMTGGCTCAG	20 bp	54.0℃	45.00%
1492 Reverse primer	CGGTTACCTTGTTACGATT	20 bp	53.0℃	45.00%

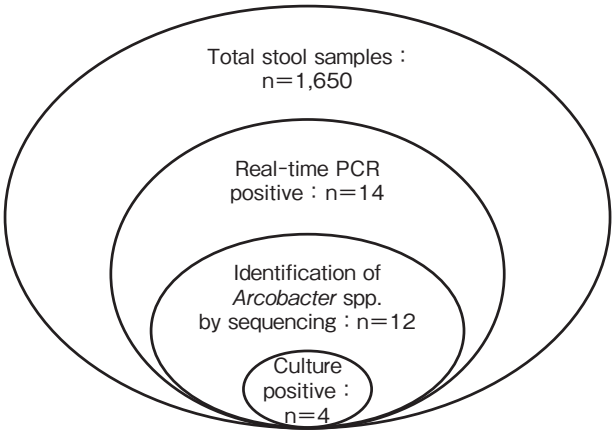

Figure-3 Summary of the results of stool-culture, real-time PCR and sequencing of PCR products for *Arcobacter* species

Number of stool samples tested in this study was 1,650, and real-time PCR was positive for 14 samples (0.8%). Among fourteen samples with positive real-time PCR results, two samples showed non-specific PCR results different from that of *Arcobcter* species, and four samples (28.6%) had positive culture results at the same time.

Table-3 Summary of the results of stool-culture, real-time PCR and sequencing of PCR products

Sample No.	Culture	PCR	Sequence analysis
1	+	+	
2	+	+	
3	+	+	
4	+	+	
5	-	+	
6	-	+	*A. butzleri*
7	-	+	
8	-	+	
9	-	+	
10	-	+	
11	-	+	
12	-	+	*A.skirrowii*
13*	-	+	unspecific
14*	-	+	

* The samples No.13 and No.14 showed positive reaction by real-time PCR but were not identified as *Arcobacter* species by sequencing.

Thermal Cycler Dice® (Takara Bio Inc., Shiga, Japan) according to manufacturer's instructions. To prove that the DNA of enteropathogenic bacteria was contained in each reaction tube, PCR was performed using primers designed to amplify 16S rRNA (Table-2).

Results

1. Identification of *Arcobacter* species using bacterial culture (Figure-3)

Of the 1,650 stool samples tested, thirteen were positive when inoculated on agar plates selective for *Campylobacter* species and incubated at 37℃ under microaerobic conditions. Of these thirteen isolates, seven were identified as *Camylobacter jejuni*, one as *C. coli*, one as *Campylobacter species*, and four (0.24%) as *Arcobacter butzleri* when analyzed by MALDI-TOF. No other bacterial pathogens causing enteritis were isolated from these four stool samples containing *Arcobacter butzleri* isolates, and no other *Arcobacter* species was detected in any of the isolates tested by MALDI-TOF.

2. Identification of *Arcobacter* species using real-time PCR (Table-3, Figure-2)

Of the 1,650 stool samples, fourteen (0.85%) showed positive reaction by real-time PCR. Sequencing of these fourteen real-time PCR products confirmed that twelve were of *Arcobacter* species. Of the twelve positive samples, eleven were identified as *A. butzleri* and one was *A. skirrowii*. Thus, twelve (0.73%) of the 1,650 stool samples tested were positive for *Arcobacter* species by real-time PCR, with this method having an accuracy of 85.7% (twelve per fourteen real-time PCR-positive samples) in detecting *Arcobacter* species[3]. Other two real-time PCR products could not be identified by sequencing and searching in leBIBI-QBPP database.

All four culture-positive samples were included in the twelve real-time PCR-positive samples for *Arcobacter* species (Figure-3). This result indicates that real-time PCR is more sensitive than culture methods in detecting *Arcobacter* species in stool samples.

Table-4 Sensitivity of real-time PCR assay using *Arcobacter buzleri* strain DSM_8739

Solution	Negative control	10^1 CFU	10^2 CFU	10^3 CFU	10^4 CFU	10^5 CFU	10^6 CFU
PBS	−	−	−	+	+	+	+
Stool	−	−	−	−	−	+	+

CFU: colony forming unit.

3. Stool culture from frozen PCR-positive stool samples

The twelve real-time PCR-positive samples were thawed and culured again. However, *Arcobacter* species did not grow up from these samples.

4. Sensitivity and specificity of real-time PCR method for *Arcobacter* species

The sensitivity of real-time PCR for *Arcobacter* species was 685 copies of PCR products per reaction when assessed using the PCR products of *A. butzleri* DSM_8739, 10^2 CFU ml^{-1} (2 CFU/reaction) when using DSM_8739 solution in PBS, 10^4 $CFUg^{-1}$ stool (50 CFU $reaction^{-1}$) when using a stool sample spiked with DSM_8739 (Table-4). Six clinical isolates of enteropathogenic bacteria, including one isolates of *C. coli* and one of *C. jejuni,* were negative for *Arcobacter* species by real-time PCR (Table-5).

Table-5 Specificity of the primers for *Arcobacter* species

	Primers for *Arcobacter* species	Primers for 16S rRNA of bacteria
Aeromonas hydrophila	−	+
Arcobacter butzleri	+	+
Campylobacter coli	−	+
Campylobacter jejuni	−	+
Escherichia coli O157	−	+
Salmonella typhimirium	−	+
Salmonella sonnei	−	+

Discussion

Culture identification of *Arcobacter* species is an essential procedure to know the clinical importance of this pathogen. This study demonstrated that a selective agar for *Campylobacter* species was also useful in detecting *Arcobacter* species in stool samples by appropriate culture condition. This selective agar contains vancomycin (10 mg l^{-1}), cefoperazone (32 mg l^{-1}) and amphotericin B (3 mg l^{-1}) to eliminate intestinal microbiota[20]. *Arcobacter* as well as *Campylobacter* species are intrinsically resistant to these antibiotics[21]. Both *Arcobacter* and *Campylobacter* species can grow at 37℃ with microaerobic condition[4) 5)]. However, it is difficult to distinguish colonies of these bacteria on blood agar plates, as both are small, white-to-gray in color, have similar microscopic features (Gram-negative curved rods) and biochemical properties except for their different aerotolerance properties. Both MALDI-TOF and PCR/DNA sequencing are fast and reliable methods for distinguish these genera as well as the identification of *Arcobacter* species[14].

The prevalence rates of enteritis due to *Arcobacter* species in human are unclear in many countries including Japan, although they have been reported to range from 0.1% to 1.5%[3]. In this study, 0.2% of samples from patients with diarrhea in Germany, as same as previous report. Standardized protocols for detecting *Arcobacter* species are necessary to determine its prevalence and causative agents among patients with enteritis.

PCR-based methods are useful for detecting *Arcobacter* species in stool samples and have been found to be more sensitive than culture methods, as shown in previous study and this study[3]. However, using PCR methods, the prevalence of *Arcobacter* species in diarrheal stool samples have been reported to range from 0.4-57.0%[4) 5) 12)]. The different prevalence rates of *Arcobacter* species among studies may result from differences in pre-test probabilities, culture conditions, including medium and temperature or from differences in the sensitivities of PCR-based methods.

The isolation of *Arcobacter* species from stool samples using culture methods requires inoculation of these samples as soon as possible onto *Campylobacter* selective agar plates. Freezing of fecal samples or storage prior to isolation of bacteria was shown to reduce recovery of *Arcobacter* species as same as this study[22]. Moreover, *A. butzleri* isolates could not be recovered from PCR-positive stool

samples after storage at 4℃ [23].

The sensitivity of real-time PCR in the present study was almost same as previous study. A real-time PCR method showed detection limits of 4.2 CFU per reaction in broth and the detection limits of real-time PCR in the present study was 2 CFU per reaction in PBS (10^2 CFU ml^{-1} PBS) [24]. Another multiplex PCR method showed a sensitivity for detecting *A. butzleri* and *A. cryaerophilus* of 10^3 CFU g^{-1} chicken skin/meat sample [25], and the sensitivity of real-time PCR in the present study was 10^4 CFU g^{-1} stool (50 CFU per reaction). The different sensitivity among study may result from differences in background material of *A. butzleri* DSM_8739. DNA extraction from stool as performed in this study is more difficult than it from broth, PBS and skin/meat, because of solid components and inhibitory compound known to be present in stool.

Our real-time PCR method has also advantages in detecting a wide range of *Arcobacter* species and may therefore be better clinically than more specific methods. Furthermore, our method has sufficient specificity. It detects *Arcobacter* species but does not detect other enteropathogenic bacteria including *C. jejuni* and *C. coli*, though unspecific amplification was observed in two clinical stool samples. For the reason listed above, our real-time PCR method demonstrated the value of screening test of stool samples for *Arcobacter* species.

The major limitation of this study was that initial screening by real-time PCR was performed for the pooled samples, containing 10 stool samples in one reaction. Sensitivity and positivity rate might be higher if each sample was individually analyzed. Another limitation of this study was that although the proportion of *Arcobacter* species is extremely rare, the number of study sample is insufficient for evaluation.

In conclusion, we were able to detect *Arcobacter* species using a routine stool culture method for isolating *Campylobacter* species, with assistance of MALDI-TOF. Our real-time PCR method showed higher positivity rate than the culture method. Our genus-specific real-time PCR method has possibility to detect a broad range of *Arcobacter* species and could be used to assess the epidemiology of infectious diseases caused by *Arcobacter* species. Further investigation of the usefulness of PCR-guided culture using fresh stool samples is necessary.

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Conflict of interests

YU reports personal fees from Taisho Toyama Pharm Co, personal fees from Astellas Pharm Inc, personal fees from MSD, personal fees from Daiichi Sankyo Co, personal fees from Torii Pharm Co, personal fees from Meiji Seika Pharm Co, personal fees from Sumitomo Dainippon Pharma Co., Ltd., personal fees from Shionogi Pharm Co, personal fees from Novo Nordisk Pharm Co, grants from St. Luke's Life Science Institute, outside the submitted work. KH reports grants from Ministry of Education, Culture, Sport, Science, and Technology, Japan, during the conduct of the study; grants from Japan Agency for Medical Research and Development, personal fees from Zenyaku Kogyo Co., Ltd., outside the submitted work. SZ is on speaker's bureau for Becton Dickinson and Merck Sharp and Dohme (MSD) and on Advisory Boards for Roche Diagnostics and MSD, outside the submitted work.

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Ethical approval

Ethical approval by the institutional review board of Heidelberg University Hospital was waived because of its study design, using bacterial isolates and stool samples unconnected with patients' personal information.

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