Identification of and Screening for Human *Helicobacter cinaedi* Infections and Carriers via Nested PCR

Kohta Oyama,^a Shahzada Khan,^a Tatsuya Okamoto,^a Shigemoto Fujii,^a Katsuhiko Ono,^a Tetsuro Matsunaga,^a Jun Yoshitake,^a Tomohiro Sawa,^a Junko Tomida,^b Yoshiaki Kawamura,^b and Takaaki Akaïke^a

Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan,^a and Department of Microbiology, School of Pharmacy, Aichi Gakuin University, Nagoya, Japan^b

Helicobacter cinaedi is the most frequently reported enterohepatic *Helicobacter* species isolated from humans. Earlier research suggested that certain patients with *H. cinaedi* infection may remain undiagnosed or incorrectly diagnosed because of difficulties in detecting the bacteria by conventional culture methods. Here, we report a nested PCR assay that rapidly detects the cytolethal distending toxin gene (*cdt*) of *H. cinaedi* with high specificity and sensitivity. Specificity of the assay was validated by using different species of *Helicobacter* and *Campylobacter*, as well as known *H. cinaedi*-positive and -negative samples. The sensitivity of detection for the *cdt* gene in the assay was 10² CFU/ml urine or 10² CFU/10⁵ infected RAW 264.7 cells. In an *H. cinaedi*-infected mouse model, the *cdt* gene of *H. cinaedi* was effectively detected via the assay with urine (6/7), stool (2/3), and blood (2/6) samples. Importantly, it detected *H. cinaedi* in blood, urine, and stool samples from one patient with a suspected *H. cinaedi* infection and three patients with known infections. The assay was further used clinically to follow up two *H. cinaedi*-infected patients after antibiotic treatment. Stool samples from these two patients evaluated by nested PCR after antibiotic therapy showed clearance of bacterial DNA. Finally, analysis of stool specimens from healthy volunteers showed occasional positive reactions (4/30) to *H. cinaedi* DNA, which suggests intestinal colonization by *H. cinaedi* in healthy subjects. In conclusion, this nested PCR assay may be useful for the rapid diagnosis, antimicrobial treatment evaluation, and epidemiological study of *H. cinaedi* infection.

Since the first report of *Helicobacter cinaedi* in 1984 (1), it has been recognized as the most commonly reported enterohepatic *Helicobacter* species isolated from both immunocompromised (21) and immunocompetent (11, 22) patients. Nonetheless, a rapid and specific assay for reliable diagnosis of *H. cinaedi* infection is still not available. *Helicobacter* species grow slowly under microaerobic conditions and are therefore difficult to detect in most laboratory diagnostic settings (10, 23). Because no current methods provide a laboratory diagnosis of *H. cinaedi* infection, previous reports of *H. cinaedi* infection mostly described the growth of bacteria in a conventional microaerobic culture (11), despite the fastidious and slowly growing nature of *H. cinaedi* on the culture media. Also, since selective media for isolation of enteric bacteria from stool specimens frequently use cephalothin and cefazolin, the growth of *H. cinaedi* is inhibited by these agents (10). Direct PCR of biological samples also has limited sensitivity because of the presence of inhibitory substances (2). We previously described an enzyme-linked immunosorbent assay (ELISA) for measuring the level of serum IgG antibody against a 30-kDa major antigenic protein of *H. cinaedi* (6, 8). Although measurement of serum IgG antibodies is useful for long-term follow-up of the host immune response to pathogens, it does not provide information about the presence of viable bacteria in the host. Among the other available methods, whole-cell protein electrophoresis (23) may be useful for species-level identification, but it also requires successful isolation of the bacteria and some time (more than a few weeks) for proper culture. Until now, the best way to confirm the diagnosis of *H. cinaedi* infection utilized PCR amplification of 16S rRNA genes of the isolated bacteria for species-level identification. However, comparison of the near-complete 16S rRNA gene sequences may have also led to incorrect identification of *H. cinaedi* in past years (23). Therefore, more

reliable, easier, and faster molecular methods are needed for diagnosis of *H. cinaedi* infection.

The recent completion of the *H. cinaedi* genome sequence (GenBank accession number AP012344.1) has provided the opportunity to develop molecular diagnostic methods based on primer sequences specific to *H. cinaedi* (5). The current study validated and applied a nested PCR assay that, with experimental and clinical specimens of different origins, targeted the cytolethal distending toxin gene (*cdt*) of *H. cinaedi* not only for rapid diagnosis of *H. cinaedi* infection but also for screening of carriers of *H. cinaedi*. We conclude that this nested PCR assay is useful for early and efficient diagnosis of human *H. cinaedi* infections and identification of *H. cinaedi* carriers.

MATERIALS AND METHODS

Bacterial culture. A clinical isolate of *H. cinaedi* (strain PAGU 0616) (5, 6, 8, 11) was used for experimental infections and optimization of *H. cinaedi* detection in various clinical specimens. DNA extracted from this isolate was used as a positive control for nested PCR assays. *Helicobacter cinaedi* bacteria were grown on *Helicobacter* agar plates (Nissui Pharmaceutical, Tokyo, Japan) under controlled microaerobic conditions in an atmosphere of 10% H₂, 10% CO₂, and 80% N₂ at 37°C for 2 to 3 days. *Helicobacter* agar plates contain vancomycin (5.0 mg/liter), cefsulodin (2.5 mg/

Received 19 June 2012 Returned for modification 28 July 2012

Accepted 11 September 2012

Published ahead of print 26 September 2012

Address correspondence to Takaaki Akaïke, takakaik@gpo.kumamoto-u.ac.jp.

K. Oyama and S. Khan contributed equally to this article.

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doi:10.1128/JCM.01622-12

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liter), trimethoprim lactate (2.5 mg/liter), and amphotericin B (2.5 mg/liter) for selective isolation of *Helicobacter*. Bacterial cultures were harvested in 10 mM phosphate-buffered saline (PBS), and inoculum doses were adjusted by measuring the optical density of the bacterial suspension at 620 nm.

Cell culture and experimental infection of cultured cells. Murine macrophage-like RAW 264 cells were maintained in Dulbecco's modified Eagle medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum in a 5% CO₂ atmosphere at 37°C. Cells were seeded in 12-well plates (2×10^5 cells/well) for 12 h and were infected with different doses of *H. cinaedi* for 1 h. After infection, cells were scraped and collected in the same medium. The cell suspension was then centrifuged for 5 min at 5,000 $\times$ g, and the resultant pellet was used for DNA extraction.

Animal infection model and sample collection. Six-week-old ddY mice were infected intraperitoneally with *H. cinaedi* at 10^8 CFU/mouse. Mice were killed at 1, 6, and 24 h postinfection (p.i.); during dissection, blood and urine samples were collected aseptically directly from the heart and the bladder, respectively. To produce an animal model of naturally occurring infection via the hepatointestinal route, we also challenged ddY mice orally once with *H. cinaedi* (10^9 CFU/mouse) suspended in 200 μ l of PBS. Stool samples were collected before and 1 day after the oral challenge. All animal experiments were carried out according to the Guidelines in the Laboratory Protocol of Animal Handling, Kumamoto University Graduate School of Medical Sciences.

Diagnosis of *H. cinaedi* infection and collection of clinical specimens. Four patients with fever and/or cellulitis after orthopedic surgery at Kumamoto Orthopedic Hospital were evaluated for *H. cinaedi* infection. Bacteria from these patients were isolated by using a previously described conventional blood culture method (11). In brief, a 10-ml aliquot of blood collected from a patient was cultured by using the BD Bactec Plus Aerobic/F system (Becton, Dickinson and Company, Franklin Lakes, NJ). A part of the culture sample that was positive for bacterial growth was inoculated onto *Helicobacter* agar under a microaerobic environment as described above. Bacterial growth on agar plates was initially characterized by evaluating colony morphology, urease testing, motility, and Gram staining. Species-level identification was carried out with 16S rRNA by means of a conventional PCR analysis described previously (11). Stool, urine, and blood samples were collected from infected patients and subjected to nested PCR for confirmation of *H. cinaedi* infection. Samples were generally collected for nested PCR on the day of onset of cellulitis and fever. For stool cultures for patients suspected of having *H. cinaedi* infections, the stool was suspended in PBS and a loopful of the suspension was inoculated onto *Helicobacter* agar and incubated as described above. Total bacterial growth on agar plates was collected with PBS and used for DNA extraction and PCR analysis as described below.

Collection of stool specimens from healthy humans without apparent *H. cinaedi* infection. Stool samples were randomly collected from 30 healthy employees (5 male and 25 female; ages ranged from 20 to 78 years, with an average of 35.2 years) working in a hospital in Kumamoto City in April 2012. Samples were kept on ice during transfer to the Department of Microbiology, Kumamoto University, and were stored at -80°C until used for DNA extraction.

Ethical compliance. The procedure for obtaining specimens from humans in this study complied with those recommended by the Regional Ethical Committee on Human Experimentation of Kumamoto University and Kumamoto Orthopedic Hospital. Written informed consent was obtained from each subject.

***Helicobacter cinaedi* DNA extraction.** Extraction of DNA from clinical samples, infected RAW 264 cells, samples obtained from experimentally infected mice, urine samples, and samples of pure bacteria in buffer was performed by using commercially available kits. The DNeasy blood and tissue minikit (Qiagen, Hilden, Germany) was used for cell, blood, and pure bacterial cultures, and the QIAamp DNA stool minikit (Qiagen) was used for urine and stool samples, according to the manufacturer's

instructions. In some experiments, 10-fold dilutions of a known stock concentration of *H. cinaedi* suspended in PBS were spiked to 100- μ l aliquots of urine samples before DNA extraction and used as an *H. cinaedi*-containing positive control for the nested PCR.

Nested PCR. PCR amplifications were performed in an Astec Program Temp Control System (Astec Inc., Fukuoka, Japan) with reagents obtained from Toyobo (Osaka, Japan). Primary reactions used 5.0 μ l of stool or urine DNA (25 to 100 ng), 2.0 μ l of blood DNA (200 to 300 ng), or 2.0 μ l of purified *H. cinaedi* DNA (approximately 0.5 ng) as the template in a total volume of 25 μ l. The PCR master mixture included deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) with each at 200 μ M, 1.25 U DNA polymerase (KOD-Plus-, Toyobo, Osaka, Japan), and each primer at 0.25 μ M. The primer sequences used for the detection of the *H. cinaedi*-specific *cdt* gene (GenBank accession numbers [ABQT01000017.1](#) and [AF243080.1](#)) by PCR were 5'-GGAGCTGTGAGTGTGCTG-3' (forward primer) and 5'-AAATGACCGACACGAGCTG-3' (reverse primer), and these primers were used to amplify a 659-bp gene sequence (18). Cycling conditions involved an initial 3-min denaturation at 95°C, followed by 35 cycles, each consisting of 10 s of denaturing at 98°C, 30 s of annealing at 58°C, and 35 s of extension at 68°C. These 35 cycles were followed by 7 min of extension at 68°C. Reaction products were then maintained at 4°C until they were used as templates for nested reactions. The primers nested within the first set, 5'-GGATTTAGGCTCTCGCTCTCGTCCGGATAT-3' (forward primer) and 5'-ATCGCCGCTCTTACTC GCGTCTCCAAATTA-3' (reverse primer), were used for a second amplification reaction and yielded a 359-bp product. Nested amplifications used 1.0 μ l of the amplicon from the first PCR as the template in a total volume of 25 μ l. Nested cycling conditions and reaction compositions were same as described above for the primary amplification. PCR amplicons were then electrophoresed with a 1% agarose gel, stained with ethidium bromide, visualized under UV light, and evaluated. For quality control, each amplification run included two negative controls, one for the PCR (only master mix) and the other for the DNA extraction (Milli-Q water instead of sample), and a positive control (2 μ l of pure genomic DNA extracted from *H. cinaedi*). Aseptic conditions and filter tips were used throughout the assay to minimize possible cross-contamination. To validate the sensitivity of the nested PCR assay, we used *H. cinaedi* isolates collected from infected patients ($n = 10$). The sensitivity of detection for the *cdt* gene in the assay was determined either using DNA extracted from urine samples that were spiked with serially diluted suspensions of *H. cinaedi* or using DNA from RAW 264.7 cells infected with different doses of *H. cinaedi*. Human samples showing positive bands in the nested PCR were reanalyzed starting from the DNA extraction step to confirm the result. For DNA sequencing of the PCR product, nested PCR products amplified from *H. cinaedi* were purified from a 1% agarose gel with a gel extraction kit (Qiagen). Nucleotide sequences of the products were determined by using a dye terminator reaction kit (Applied Biosystems, Foster City, CA) and an automatic sequencer (ABI Prism 310; Applied Biosystems). In our preliminary studies, *H. cinaedi* DNA could not be detected in clinical samples (e.g., blood and stool) collected from *H. cinaedi*-infected patients by using only the first PCR of *cdt* gene. Therefore, we used the nested PCR to detect *H. cinaedi* DNA with clinical samples.

Phylogenetic analysis. Phylogenetic analysis of clinical isolates of *H. cinaedi* was performed by using the 16S rRNA sequence as described previously (11). Briefly, 16S rRNA genes of *H. cinaedi* isolates were amplified by PCR as previously described (7, 13). The sequences were determined by means of an automatic sequencer (model 3100; Applied Biosystems) and the dye terminator reaction kit. For each strain, about 1,430 bp of the 16S rRNA gene sequence was determined. To detect closely related species, each sequence found was analyzed by using the FASTA search system (15) included at the DNA Data Bank of Japan (DDBJ) website (<http://www.ddbj.nig.ac.jp>). Sequences of the 16S rRNA genes of closely related species of *Helicobacter* were taken from the DDBJ, GenBank, and European Molecular Biology Laboratory databases. CLUSTAL-X software, originally described by Thompson et al. (20), was used to determine the phy-

FIG 1 Design and specificity of nested PCR. (A) Arrows indicate the locations of PCR primers in the whole genome sequence, and the sizes of the products of the primary and nested amplification reactions are shown. (B) Nested PCR amplification of bacterial DNA. Lane 1, 1-kb marker; lane 2, standard clinical isolate of *H. cinaedi*; lane 3, master mix without template; lane 4, Milli-Q water control for DNA extraction; lanes 5 to 14, *H. cinaedi* clinical isolates from 10 patients; lane 15, *H. pylori* PAGU 151^T (ATCC 43504^T); lane 16, *H. hepaticus* PAGU 604^T (LMG 16316^T). (C) First PCR amplification of the *cdt* gene of *H. cinaedi*. Lane 1, 100-bp DNA ladder; lane 2, *H. cinaedi* PAGU 597^T (CCUG18818^T); lane 3, *H. fennelliae* PAGU 601^T (LMG 18294^T); lane 4, *H. pametensis* PAGU 607^T (LMG 12678^T); lane 5, *H. acinonychis* PAGU 609^T (LMG 12684^T); lane 6, *H. muridarum* PAGU 606^T (LMG 13646^T); lane 7, *H. hepaticus* PAGU 604^T (LMG 16316^T); lane 8, *H. bilis* PAGU 599^T (LMG 18386^T); lane 9, *H. pylori* PAGU 151^T (ATCC 43504^T); lane 10, *H. canadensis* PAGU 600^T (CCUG 47163^T); lane 11, *H. canis* PAGU 598^T (NCTC 12379^T); lanes 12 to 14, clinical isolates of *H. cinaedi*; lane 15, *C. fetus* subsp. *fetus* PAGU 74^T (ATCC 27374^T). (D) Sensitivity of detection of the *H. cinaedi*-specific *cdt* gene by nested PCR. Serial titrations of pure *H. cinaedi* culture were performed, samples were mixed with 10⁶ RAW 264 cells, and then nested PCR was performed by using the extracted DNA of the mixture of bacteria and cells (left). Tenfold dilutions of pure bacterial culture were spiked into healthy human urine, and nested PCR was performed by using the extracted DNA from contaminated urine (right).

logenetic relationships for each isolate. TreeView software was used to draw the phylogenetic tree (14, 15).

RESULTS

Design, specificity, and sensitivity of the nested PCR. The *H. cinaedi* nested PCR utilized a primer pair in the primary reaction to amplify a 659-bp sequence of the *cdt* gene of *H. cinaedi*, followed by a second reaction with a different primer pair to amplify a 359-bp sequence within the primary amplification product. Figure 1A shows the locations of these primers in the *cdt* gene. DNAs extracted from all clinical isolates ($n = 10$) of *H. cinaedi* gave a single amplification product of 359 bp after the nested PCR. The nested PCR produced no amplification when DNAs obtained from *Helicobacter pylori* (ATCC 43504) and *Helicobacter hepaticus* (PAGU604) were used as templates (Fig. 1B). None of the DNA samples extracted from other *Helicobacter* species tested (*H. fennelliae*, *H. pametensis*, *H. acinonychis*, *H. muridarum*, *H. hepaticus*, *H. bilis*, *H. pylori*, *H. canadensis*, and *H. canis*) and *Campylobacter fetus* subsp. *fetus* GTC 260^T were amplified by the first PCR assay (Fig. 1C). The sensitivity of this nested PCR detection was evaluated by using DNA samples extracted from *H. cinaedi*-infected RAW 264 cells and from *H. cinaedi*-contaminated urine samples.

The detection limit thus determined was 10 CFU/10⁵ RAW 264 cells and 10 CFU/100 μ l of urine (Fig. 1D).

Nested PCR with specimens from *H. cinaedi*-infected mice.

Nested PCR analysis was performed on blood and urine specimens obtained from intraperitoneally infected mice and on stool specimens collected from mice after oral administration of *H. cinaedi*. Nested PCR amplified DNA extracted from the blood of one mouse (of three) at 1 h p.i. and another mouse at 6 h p.i. DNA extracted from the blood at 24 h p.i. produced no amplification signal (Fig. 2A). DNA from urine, however, showed a positive PCR more frequently (six of seven samples) throughout the time course of infection than did DNA extracted from the blood samples (Fig. 2B). DNA from stool samples collected before infection with bacteria did not show the 359-bp product with the nested PCR. DNA from two stool samples collected even 7 days after oral administration of bacteria, however, showed amplification with nested PCR (Fig. 2C).

Clinical history of *H. cinaedi* infection and laboratory diagnosis of infection with nested PCR. Nested PCR was performed with DNA extracted from clinical specimens that were collected from patients at different time points starting from the date of onset of fever. Table 1 provides the clinical characteristics of the

FIG 2 Nested PCR with experimental samples collected from *H. cinaedi*-infected mice. (A and B) Nested PCR amplification of DNA extracted from blood (A) and urine (B) samples from intraperitoneally infected mice at the indicated time points. (C) Nested PCR amplification of DNA extracted from stool samples from uninfected control and orally infected mice. Stool samples were collected at 7 days p.i. Lanes 1, 2, and 3, DNA extracted from three different mice.

patients. Patient 1 had recurrent bacteremia with *H. cinaedi*. During the first occurrence of bacteremia, this patient received combination therapy with piperacillin (2 g/day) and minocycline (200 mg/day) (by intravenous drip infusion) for 7 days and with only minocycline for 11 days (oral), and he recovered. As Fig. 3A illustrates, 4 months after his recovery from fever and cellulitis, this patient was readmitted because of pyogenic spondylitis at the lumbar vertebrae (L4/5), and his blood culture was positive for *H. cinaedi*. DNA from the stool sample collected at the day of latest onset of pyogenic spondylitis showed the 359-bp band with nested PCR (Fig. 3B). The stool sample was also inoculated onto culture plates, which clearly manifested formation of bacterial colonies that resembled those of a type strain of *H. cinaedi* (Fig. 3C). DNA from bacteria grown on the stool culture plates also showed the 359-bp band by nested PCR. Nested PCR using DNA from blood and urine samples collected at the day of latest onset of pyogenic spondylitis produced no positive bands, although blood culture revealed *H. cinaedi* bacteria after positive gene amplification (Fig. 3B). Patient 2 underwent arthroplasty on the right knee and developed a high temperature with cellulitis of the right lower leg 24 days after this orthopedic operation. This patient's blood culture was positive for bacterial growth. The nested PCR did not amplify the DNA from blood, but DNA from urine produced the 359-bp band on the day of onset of cellulitis and 3 days after the onset (Fig. 3D). DNA from urine collected 5 days after the onset of cellulitis showed negative nested PCR results. DNA from the blood of patient 3 showed positive amplification on the fourth day after the onset of fever, and DNA from urine did so on the second and seventh days after the onset of fever (Fig. 3E); no positive blood culture was obtained, however.

TABLE 1 Clinical characteristics of patients

Characteristic	Patient 1			Patient 2			Patient 3			Patient 4		
	Primary infection	Recurrent infection		Primary infection	Recurrent infection		Primary infection	Recurrent infection		Primary infection	Recurrent infection	
Age (yr)	73			80			66			81		
Sex	Male			Female			Male			Female		
Orthopedic diagnosis	Left shoulder rotator cuff tear			Right knee osteoarthritis			Lumber disc herniation			Fracture of left radius and ulna		
Date of orthopedic surgery	2 November 2010			30 November 2010			26 November 2010			16 August 2011		
Date of onset of cellulitis and/or fever	24 November 2010			24 December 2010			6 December 2010			22 August 2011		
Location of cellulitis	Left shoulder			Right lower leg			Not observed			Not observed		
Hospital stay (days)	39			30			—			—		
White blood cell count (cells/mm ³)	12,180			8,000			10,970			12,500		
C-reactive protein (mg/dl)	10.4			2.8			9.8			5.1		
Stool culture	+			+			Not tested			Not tested		
Blood culture	+			+			—			+		
PCR (blood culture)	+			+			—			+		
Antibiotic treatment	Piperacillin, minocycline			Doripenem, minocycline			Doripenem, minocycline			Doripenem		
		Sulbactam, ampicillin, minocycline, levofloxacin								Minocycline		

^a —, not available or not tested.

FIG 3 Nested PCR with clinical specimens collected from patients with known or suspected *H. cinaedi* infections. Patients 1 and 2 had cellulitis, fever, and positive blood tests for growth of anaerobic bacteria that were subsequently confirmed to be *H. cinaedi* by conventional PCR. (A) Brief clinical history of patient 1. WBC, white blood cells; div, intravenous drip infusion; CRP, C-reactive protein. (B) Nested PCR with DNA from the indicated specimens from patient 1. (C) Left, morphological appearance of bacterial growth on *Helicobacter* agar plates inoculated with a stool specimen from patient 1. Plates were incubated for 4 days at 37°C in a microaerobic atmosphere containing 10% H₂ and 10% CO₂. Right, bacterial morphology of an isolate from culture of stool from patient 1 (acridine orange staining). (D and E) Nested PCR with DNA from the indicated specimens from patient 2 (D) and patient 3 (E). Patient 3 had cellulitis and fever, but a blood test was negative for anaerobic bacterial growth.

Nested PCR with stool specimens to monitor the clearance of *H. cinaedi* after antibiotic treatment. We then performed nested PCR to detect bacterial DNA in patients who had had antibiotic treatment. Because of the probable presence of PCR-inhibitory materials in DNA extracted from stool samples, we initially varied the amounts of amplicon from the primary PCR as templates to amplify the 359-bp sequence in the second PCR. Among three different amounts ranging from 0.1 to 5.0 µl, 1.0 µl of the first PCR product produced the most appreciable and consistent detection of *H. cinaedi* DNA in the stool samples of patient 4 (Fig. 4A). This patient developed a fever 6 days after having orthopedic surgery. *Helicobacter cinaedi* infection was confirmed a week after the onset of fever, and the patient received the antibiotics sulbactam and ampicillin for 3 days and thereafter doripenem (intravenous drip infusion) for 15 days and was discharged from the hos-

pital. This patient developed cellulitis of the lower legs at 65 days postoperation (p.o.), and minocycline was administered orally for 18 days (from 72 to 90 days p.o.). Nested PCR with randomly collected samples after this therapy showed the presence of bacterial DNA in stool until 72 days p.o., with results becoming negative after minocycline treatment after 72 days p.o. (Fig. 4A). Stool samples from patient 1 were also analyzed by nested PCR for a follow-up period after antibiotic therapy (Fig. 4B). As mentioned above, patient 1 had a recurrent infection with *H. cinaedi* and was treated with sulbactam and ampicillin (intravenous drip infusion for 30 days and orally for 8 days) and with minocycline and levofloxacin (orally) for 34 days, at the latest period of infection. Nested PCR was performed with stool DNA at the time points of 28, 38, and 72 days after the initiation of antibiotic treatment. Stool samples were also cultured on *Helicobacter* agar plates. DNA

FIG 4 Follow-up of *H. cinaedi*-infected patients by means of nested PCR of DNA from stool and blood specimens during antibiotic treatment. (A) Patient 4. Various amounts of products from the first PCR were used as templates for the nested PCR. The diffuse signal in the lane of the 5.0-µl template, at 90 days p.o. for the blood DNA, was due to nonspecific PCR products caused by too much template DNA being used. The lower panel gives a brief clinical and therapeutic history. Arrowheads indicate times of collection of stool or blood samples for bacterial culture and DNA amplification. (B) Patient 1. Samples were inoculated onto *Helicobacter* agar plates, and suspected colonies from the resultant bacterial growth were subcultured to obtain pure *H. cinaedi* cultures (Fig. 3C). Nested PCR was performed with DNA from both the stool specimen and the stool culture. The lower panel gives a brief clinical and therapeutic history of recurrent infection. Arrowheads indicate times of collection of stool samples. The nucleotide sequences of all PCR products from patients were consistent with that of the *H. cinaedi* type strain.

FIG 5 Genetic analysis of clinical isolates of *H. cinaedi*. The 16S rRNA gene was analyzed by means of the FASTA search system. Phylogenetic relationships for various clinical isolates recently obtained, mainly in Japan (the isolation location is given in parentheses), and *H. bilis* were analyzed on the basis of the 16S rRNA gene sequence (1,430-bp area). The *Helicobacter cinaedi* strains isolated from patient 1 from the primary and the recurrent infections (indicated in the dashed box; PAGU1625-Kumamoto and PAGU1679-Kumamoto) were found to be almost genetically identical on the basis of the 16S rRNA gene sequence. Numbers at nodes are percent occurrence in 1,000 bootstrapped trees.

from the stool culture and stool samples collected 28 and 38 days after initiating treatment was amplified by nested PCR, but the stool DNA collected after 72 days was not amplified (Fig. 4B). Growth of *H. cinaedi* on *Helicobacter* agar inoculated with stool specimens collected after 72 days was also negative (data not shown). These patients did not suffer recurrence (cellulitis or fever) after the clearance of *H. cinaedi* by antibiotic treatment, at least during a 1-month observation period.

Phylogenetic analysis of clinical isolates. Two blood culture isolates obtained during recurrent bacteremia of patient 1 were used along with 28 other clinical isolates and the type strain of *H. bilis* for phylogenetic analysis based on 16S rRNA gene sequences to determine the type of *H. cinaedi* strains causing recurrent infection of patient 1. Two isolates causing recurrent bacteremia in patient 1 at two different time points belonged to the same cluster in phylogenetic analysis. These isolates were distant by 20 to 27 bp in a 1,430-bp area of the 16S rRNA genes from the other major clinical strains causing bacteremia in the same hospital from 2004 to 2008 (Fig. 5). We considered from this observation that some number of organisms of this *H. cinaedi* strain could have survived in patient 1 after antibiotic therapy for the first occurrence of bacteremia and might possibly have caused the second instance of bacteremia.

Potential application of nested PCR for screening *H. cinaedi* carriers. We sought to develop a screening tool for possible *H. cinaedi* carriers in the natural and hospital environments. Stool specimens from apparently healthy volunteers ($n = 30$) were ex-

amined for *H. cinaedi* DNA amplification by the nested PCR assay, which revealed four positive reactions (13.33%). The nucleotide sequences of all positive PCR products from these volunteers were consistent with that of the *H. cinaedi* type strain. Although more extensive epidemiological study is required to determine the exact spread of this bacterium in general human populations, this preliminary work suggests that there may be asymptomatic human carriers who may be the source of *H. cinaedi* infection in humans.

DISCUSSION

In this study, we describe a sensitive and specific nested PCR assay for diagnosis of *H. cinaedi* infection of humans. Diagnosis of *H. cinaedi* in microbiological laboratories is difficult because of *H. cinaedi*'s fastidious growth requirements and low growth rate. Laboratory growth of *H. cinaedi* requires a microaerobic atmosphere containing 5 to 10% H_2 and 10% CO_2 , a gas mixture that is not commercially available (2). The use of nested PCR with urine or stool DNA extracts resolves these technical laboratory difficulties and may provide a more rapid diagnosis than with the conventional culture method (8 h versus 1 week). In addition, with the recently available improved PCR reagents and efficient commercial kits for DNA extraction, sensitivities of PCR methods appear to surpass those of conventional culture methods (16). The nested PCR described here used a simple and commercially available kit for DNA extraction, which made it amenable to routine use in diagnostic laboratories worldwide. To avoid false-positive

and -negative signals, this nested PCR required the regular use of positive and negative controls for both DNA extraction and PCR amplification; in addition, proper PCR techniques and aseptic conditions must be maintained.

The frequency of DNA detection varied greatly for the different kinds of biological samples used for DNA extraction and nested PCR: urine and stool samples provided better detection than blood samples (Fig. 2 and 3). This finding suggests that *H. cinaedi* bacteria are rapidly cleared from the blood circulation of the host. The use of bacterial cultures of various clinical specimens under appropriate laboratory conditions should logically increase the chance of identification of *H. cinaedi* by nested PCR, but the time required for diagnosis of infection also increases (to more than 2 days). On the basis of the observations reported in this study, using DNA from stool and urine samples in addition to DNA from blood from patients with suspected infection may be the ideal option for the *H. cinaedi* nested PCR.

We previously reported a series of 11 cases of *H. cinaedi* infection of apparently healthy postoperative patients (11). During that study, many patients presented with fever and cellulitis, which are typical symptoms of *H. cinaedi* infection, but could not be diagnosed properly because the bacteria could not be isolated by a conventional culture method using blood and stool samples. For example, patient 3 described in this study had the typical symptoms of *H. cinaedi* infection, but the conventional culture method detected no bacteria. However, the newly developed nested PCR identified *H. cinaedi* DNA in blood and urine samples collected from this patient (Fig. 3E). This finding indicates that this nested PCR is more reliable than the conventional culture method.

As was the case for the *H. cinaedi*-infected patients documented in our earlier report (11), the sources and routes of infection of our study patients here are yet to be clarified. The recurrent infection of patient 1 in this study with strains of *H. cinaedi* having identical 16S rRNA gene sequences (Fig. 5) suggests a possible relapse of the latent strain in this patient. Human infections may therefore be naturally caused by endogenous, colonizing *H. cinaedi* bacteria. Identifying the sites and mechanisms of survival of *H. cinaedi* in infected patients is of great importance. The isolation of bacteria from stool or detection of bacterial DNA from stool and urine specimens from the patients described here suggests colonization of the bacteria in the enterohepatic systems of these patients. This finding may also indicate a fecal-oral route for transmission of this bacterium. The initial source of infection, however, remains unknown. *Helicobacter cinaedi* was previously isolated from the enterohepatic tracts of rhesus monkey (3) and hamsters (4). It was also recovered from human stool (9, 19) and dog feces (17). Therefore, the stool DNA amplification assay has been confirmed as a useful tool for screening individuals suspected of having *H. cinaedi* colonization or infection.

Although the last 2 decades have seen increasing numbers of reports of recurrent *H. cinaedi* bacteremia, infection with *H. cinaedi* may be more prevalent than previously estimated (11, 21) because of the considerable difficulties with the conventional culture method for proper diagnosis. Lack of a sensitive diagnostic method may also be a major reason for our poor understanding of the clinical manifestations and pathogenesis of these infections. In a recent study, we reported that serum anti-*H. cinaedi* IgG levels were high in patients with atrial arrhythmias, and we found *H. cinaedi* antigens in aortic tissues of patients with atherosclerosis (8). *Helicobacter cinaedi* organisms have also been isolated from a

patient with myopericarditis (12), which suggests a possible cardiovascular tropism of this pathogen. The successful detection of *H. cinaedi* DNA in healthy volunteers in this study may support the presence of this bacterium in the healthy human population. Whether *H. cinaedi* is part of the normal flora or is causing asymptomatic infection of these individuals is yet to be clarified, however. In view of all these observations, the nested PCR reported here as a reliable and sensitive method may aid further investigation of *H. cinaedi* and its pathogenesis and epidemiology.

In conclusion, our present nested PCR assay is a highly sensitive and specific tool for the early diagnosis of *H. cinaedi* infections and for the screening of human carriers. Therefore, it can serve as a powerful tool for clarifying the epidemiological features of *H. cinaedi* and clinical features of *H. cinaedi* infection and for defining the range of diseases associated with this emerging pathogen.

ACKNOWLEDGMENTS

We thank Judith B. Gandy for her excellent editing of the manuscript. We gratefully thank N. Misawa, A. Okayama, T. Hayashi, and S. Higashi for their invaluable discussion and provision of clinical specimens and data.

This work was supported in part by a Grant-in Aid for Challenging Exploratory Research (grant number 21659109) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. English editing of the manuscript was supported by the Advanced Education Program for Integrated Clinical, Basic and Social Medicine, Graduate School of Medical Sciences, Kumamoto University, sponsored by MEXT.

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