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Multicenter Study To Evaluate Bloodstream Infection by *Helicobacter cinaedi* in Japan▽

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***Helicobacter cinaedi* has being recognized as an important human pathogen which causes bloodstream infections. Although the first case of bacteremia with this pathogen in Japan was reported in 2003, the true prevalence of *H. cinaedi* as a pathogen of bloodstream infections in this country is not yet known. Therefore, the aim of our study was to assess the incidence of bacteremia with *H. cinaedi* in Japan. We conducted a prospective, multicenter analysis in 13 hospitals during 6 months in Tokyo, Japan. Among positive blood cultures from 1 October 2003 to 31 March 2004, isolates suspected of being *Helicobacter* species were studied for further microbial identification. Identification of the organisms was based on their biochemical traits and the results of molecular analysis of their 16S rRNA gene sequences. A total of 16,743 blood culture samples were obtained during the study period, and 2,718 samples (17.7%) yielded positive culture results for coagulase-negative staphylococci. Among nine isolates suspected to be *Helicobacter* species, six isolates were finally identified as *H. cinaedi*. The positivity rate for *H. cinaedi* in blood culture was 0.06% of total blood samples and 0.22% of blood samples with any positive culture results. All patients with bacteremia with *H. cinaedi* were found to have no human immunodeficiency virus (HIV) infection, but many of them had complications with either malignancy, renal failure, or a history of surgical operation. Therefore, our results suggest that bacteremia with *H. cinaedi* is rare but can occur in compromised hosts other than those with HIV infection in Japan.**

A growing number of *Helicobacter* species are increasingly being recognized as important human pathogens. Although the majority of helicobacters have been associated with infection of the gastrointestinal tract, several recently described helicobacters have been isolated from human blood. One of the important organisms among these may be *Helicobacter cinaedi*, which causes enteric or bloodstream infections. *H. cinaedi* was commonly isolated from homosexual men infected with human immunodeficiency virus (HIV). Later it was also isolated from other immunocompromised patients.

In 2003, the first case of bacteremia with *H. cinaedi* in Japan was reported by Murakami et al. (10). The patient was HIV negative but was receiving immunosuppressive therapy after renal transplantation. However, as far as we know, the true prevalence of this organism as a pathogen causing bacteremia in Japan is still not yet known. We therefore performed a prospective laboratory-based multicenter study, and we describe the diagnostic problems involved in identifying the causative organism.

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MATERIALS AND METHODS

Surveillance. We performed prospective laboratory-based multicenter surveillance in 13 hospitals located in Tokyo, Japan, from 1 October 2003 to 31 March 2004. All hospitals had automated blood culture systems (BACTEC; BD Diagnostic Systems, Tokyo, Japan), and all provide medical care to adults and children in different medical specialties. The research protocol was approved by the local institutional review board of each site.

Blood culture microbial identification. Blood was collected in BACTEC culture bottles and incubated in a BACTEC 9050 blood culture system for at least 4 days (Becton Dickinson, BD Biosciences, Tokyo, Japan). Positive blood culture samples were further investigated for the microbial identification. To identify the bacterial isolates, phenotypic tests commonly used to characterize helicobacters were performed. Growth was examined under aerobic, microaerobic, and anaerobic conditions at 35°C. Bacteria were Gram stained and tested for urease activity by using a selective rapid urea test. Dihydrogen sulfide production, γ-glutamyltransferase activity, hippurate hydrolysis, and nitrate reduction were determined by using the Campy identification system (bioMerieux Vitek, Tokyo, Japan). Some currently available biochemical tests have been reported to be unable to conclusively identify or distinguish *H. cinaedi* from other fastidious *Campylobacter* species or *Helicobacter* species (13). Therefore, we decided to examine both suspected *Campylobacter* isolates and suspected *Helicobacter* species for further identification.

Patients. Patients with *H. cinaedi* bacteremia were reviewed to determine the clinical background, the source of infection, and the outcome. The case report form contained the following information: age, gender, date of admission, ward, date of bacteremia, underlying medical conditions, exposure to invasive medical procedures, use of antibiotics or corticosteroids, management of bacteremia (antimicrobial treatment, catheter removal), and outcome.

Amplification of 16S rRNA. Bacteria were grown on blood agar plates for 48 h, and chromosomal DNA was prepared using hexadecyltrimethyl ammonium bromide as described previously (17). PCR of the 16S rRNA gene was performed with the primers 8F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 15R (5′-AAG GAG GTG ATC CAR CCG CA-3′), which were designed based on the

TABLE 1. Results of blood cultures and *H. cinaedi* positivity

Institution	Total no. of samples	No. (%) of culture-positive samples	No. (%) of *H. cinaedi*-positive samples
Nihon University Itabashi Hospital	2,706	430 (15.9)	2 (0.074)
Surugadai Nihon University Hospital	299	59 (19.7)	0 (0)
Showa University Hospital	1,417	247 (17.4)	0 (0)
Toho University Omori Medical Center	1,200	162 (13.5)	1 (0.083)
Teikyo University Hospital	1,084	192 (17.7)	0 (0)
Tokyo Medical and Dental University	966	102 (10.6)	0 (0)
Tokyo Metropolitan Geriatric Hospital	1,154	187 (16.2)	0 (0)
Toranomon Hospital	2,860	310 (10.8)	3 (0.104)
Mitsui Memorial Hospital	750	124 (16.5)	0 (0)
Nakano Sogo Hospital	454	113 (24.9)	0 (0)
Toshiba General Hospital	465	66 (14.2)	0 (0)
Byotai Seiri Laboratory	3,089	630 (20.4)	0 (0)
Tokyo Koseinenkin Hospital	299	96 (32.1)	0 (0)
Total	16,743	2,718 (17.7)	6 (0.036)

Escherichia coli 16S rRNA numbering system. The PCR was performed in a DNA thermal cycler (PE Applied Biosystems Division, Foster City, CA) in reaction mixtures of 25 μl containing 20 ng of genomic DNA, 2.5 μl of 10-fold-concentrated reaction buffer (QIAGEN, Inc., Tokyo, Japan), 160 nM (each) primer, 0.625 U of *Taq* DNA polymerase (QIAGEN), and 250 μM (each) deoxynucleotide (Amersham-Pharmacia Biotech, Tokyo, Japan). Samples were incubated at 94°C for 2 min to denature the target DNA. They were then cycled 30 times at 94°C for 1 min and annealed at 55°C for 1 min and 72°C for 2 min, with a final incubation at 72°C for 10 min to complete the extension.

16S rRNA data analysis. The fragment for sequencing was amplified by PCR, and the product was purified with a QIAquick PCR purification kit (QIAGEN). The nucleotide sequence was determined directly from the PCR fragment with a PCR-based reaction using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems Division) and analyzed using the PE Applied Biosystems 310 DNA sequencer (PE Applied Biosystems Division) at the Miyazaki University Gene Research Center. To determine the central region of the 16S rRNA fragment, the primers MF (5′-AAT ATT GCG CAA TGG GGGAA-3′) and MR (5′-GGC CAT GAT GAC TTG ACG TC-3′) were used for sequencing (9). Computer analyses of the DNA sequences were performed using Genetics Computer Group programs; database similarity searches were performed through the National Center for Biotechnology Information using the BLASTX algorithm.

Similarity matrices were constructed from the aligned sequence, and a phylogenetic tree was constructed based on the 16S rRNA gene sequences using the unweighted pair-group method with arithmetic averages with Genetyx-win software (version 5.0.4).

RESULTS

Positivity rate of blood cultures. A total of 16,743 blood culture samples were obtained during the study period, and 2,718 samples (17.7%) yielded positive culture results with various organisms containing coagulase-negative staphylococci. The positivity rate of blood culture including all types of bacteria in each institution ranged from 10.6 to 32.1% (average, 17.7%) (Table 1). Among these positive blood cultures, nine isolates were suspected to be *Helicobacter* and other related organisms.

Bacterial identification according to biochemical characteristics. All nine *Helicobacter* and other related isolates were found to be gram-negative spiral rods, and urease was negative. However, three of nine isolates were suspected to be *Campylobacter* spp. according to the positive growth at 25°C and 42°C and on charcoal cefoperazone deoxycholate agar (Table 2). Six other isolates were biochemically more consistent with *Helicobacter* than with *Campylobacter* based on a failure to hydrolyze hippurate, to reduce nitrate, or to grow at 42°C; we were unable to identify the species of these isolates based on these biochemical characteristics.

Identification of organisms based on 16S rRNA sequences. Identification results for isolates based on the 16S rRNA sequences are shown in Table 3. The 16S rRNA gene sequences of each suspected *H. cinaedi* strain, strains 5, 9, 11, 12, 14, and 16, and *H. cinaedi* reference strain ADN0413 revealed a high level of similarity (98 to 99%). Furthermore, the 16S rRNA gene sequences of each suspected *Campylobacter* strain, no. 4, 13, 15, and 17, and of *Campylobacter fetus* subsp. *fetus* reference strains also revealed a high level of similarity (98 to 99%).

Sequence homology among *H. cinaedi* isolates. A phylogenetic tree of the isolates was constructed based on the nucleotide sequences of the 16S rRNA genes (Fig. 1). The analysis demonstrates a high degree of sequence homology among the strains isolated.

Incidence of *H. cinaedi* bacteremia and clinical characteristics. We finally detected six cases of bacteremia due to *H. cinaedi* during the surveillance study. The patients with *H. cinaedi* bacteremia were detected in only three hospitals (23%) of the total number of participating institutions. The rate of *H. cinaedi* bacteremia in the 13 institutions ranged from 0 to 0.104%. The overall rate of *H. cinaedi* bacteremia was 0.036% of all blood culture samples (Table 1).

Clinical characteristics. The clinical characteristics of the six cases are shown in Table 3. All patients except one (83%) were female, and the median age was 52 years (range, 17 to

TABLE 2. Characteristics of suspected *H. cinaedi* isolates and other, related organisms

Isolate no.	Institution	Growth at 25°C/42°C[a]	Growth on CCDA agar[a,b]	Suspected identification by culture results	16S rRNA sequence analysis		
					% Similarity (primer)	GenBank accession no.	Identification
4	Toho University Omori Medical Center	+/+	+	*C. fetus*	98 (middleF)	AF550619.1	*C. fetus* subsp. *fetus*
5	Toranomon Hospital	−/+	−	*H. cinaedi*	98 (middleF)	M88150.2	*H. cinaedi*
9	Toho University Omori Medical Center	−/+	−	*H. cinaedi*	99 (middleF)	M88150.2	*H. cinaedi*
11	Toranomon Hospital	−/+	−	*Helicobacter* sp.	98 (middleF)	M88150.2	*H. cinaedi*
12	Nihon University Itabashi Hospital	−/−	−	*H. cinaedi*	99 (8F + middleF)	HECRR16SAE	*H. cinaedi*
13	Toho University Omori Medical Center	+/+	+	*C. fetus*	98 (middleF)	AF550619.1	*C. fetus subsp fetus*
14	Nihon University Itabashi Hospital	−/−	−	*Helicobacter* sp.	99 (8F+middleF)	HECRR16SAE	*H. cinaedi*
15	Surugadai Nihon University Hospital	+/−	+	*C. fetus*	99 (middleF)	AF550619.1	*C. fetus subsp fetus*
16	Toranomon Hospital	−/−	−	*Helicobacter* sp.	98 (middleF)	HECRR16SAE	*H. cinaedi*

[a] +, growth; −, no growth.
[b] Charcoal cefoperazone deoxycholate agar (CCDA) was a specially designed medium for the isolation of *Campylobacter* spp., and isolates were incubated microaerobically at 42°C for 40 to 48 h.

TABLE 3. Background data for patients with *H. cinaedi* bacteremia

Isolate no.	Date of sampling[a]	Age	Gender[b]	Underlying disease	Immunosuppressant drugs
5	2003/11/8	69	M	Chronic renal failure (on dialysis); mycosis fungoides	None
9	2003/11/27	69	F	Primary malignant bone tumor in lower extremity	None
11	2004/1/6	17	F	Myelodysplastic syndrome	None
12	2003/12/8	71	F	Endometrial cancer	Cisplatin, adriamycin
14	2004/2/24	24	F	Threatened premature delivery, Cesarean section	None
16	2004/3/11	63	F	Chronic renal failure (on dialysis)	None

[a] Year/month/day.
[b] M, male; F, female.

71 years). At the time of diagnosis of bacteremia, all patients were hospitalized. Underlying diseases of the patients were as follows: cancer or hematologic disorder was documented for three patients (50%), and one of these patients was undergoing chemotherapy with immunosuppressant drugs. Two other patients were undergoing hemodialysis for chronic renal failure. Another patient underwent a cesarean section because of threatened premature delivery. All patients with *H. cinaedi* bacteremia had fever, but colitis was not seen in any of these patients. Neutropenia was present in only one case (16.6%). Bacteremia was generally a late complication during the hospital stay. All patients recovered after antimicrobial therapy, including expanded-spectrum cephalosporin and carbapenem.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this study have been deposited in GenBank under the accession numbers listed in Table 2.

DISCUSSION

Many *Helicobacter* species that are known to cause diarrhea in humans also have been isolated from blood. Of these, *H. cinaedi* is the most frequently reported organism. *H. cinaedi* bacteremia occurs primarily in immunocompromised hosts, particularly in men infected with HIV. The number of HIV-seropositive persons and AIDS patients is increasing from year to year, especially among homosexual men, in Japan. In 2005, 741 HIV-infected Japanese and 302 AIDS patients were newly recorded in Japan, and the cumulative number of HIV-infected and AIDS patients until recently was reported as 11,036 cases (1). These results suggest that HIV infection is also spreading faster in Japan. However, the official reports show that HIV infection rates in this country still remain low compared to those in other countries. Therefore, we speculate HIV infection may not become a prime risk of *H. cinaedi* bacteremia in Japan. The first case of *H. cinaedi* bacteremia in Japan was HIV negative, but he was receiving immunosuppressive therapy after renal transplantation. These results suggest that there may be possible risks of *H. cinaedi* bacteremia other than that with HIV infection.

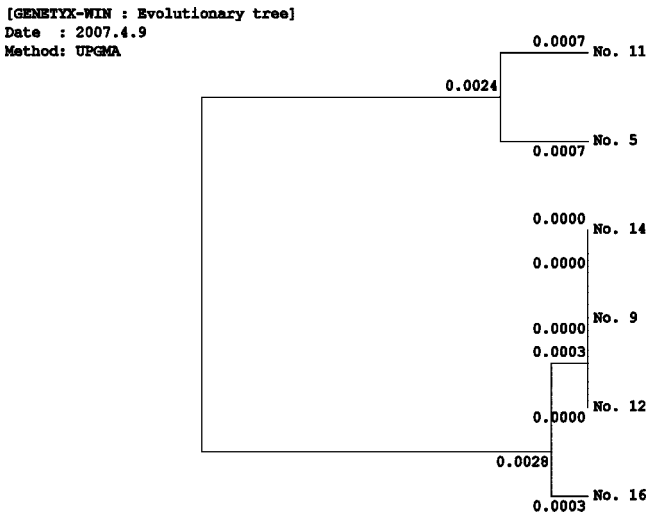

FIG. 1. Phylogenetic tree based on 16S rRNA gene sequences. High degrees of sequence homology among the strains are demonstrated.

Recently Kitamura et al. (5) reported 11 cases of *H. cinaedi* bacteremia and cellulitis that occurred consecutively during a particular period in the same hospital. Interestingly, as described in their report, no patient had any underlying immunocompromising conditions or had been given immunosuppressive agents. However, in our study most of our cases of *H. cinaedi* bacteremia had been found to have some underlying immunocompromising disease or the patients were undergoing chemotherapy with immunosuppressant drugs. We cannot clearly explain the discrepancy between these studies, but these results suggest that *H. cinaedi* should not be regarded as simply an opportunistic pathogen.

H. cinaedi is a fastidious organism, rendering microbiological diagnosis difficult. It rarely grows on traditional culture media. Growth may sometimes be obtained on rich, nonselective media (blood or chocolate agar) incubated in a microaerobic (5% O_2) atmosphere at 35°C. It is well documented that the biochemical identification of *Helicobacter* strains based on a limited number of tests is difficult, since these isolates frequently exhibit unusual phenotypic profiles within the same species (12, 14). Therefore, molecular techniques have been used to determine the nucleotide sequence of the rRNA gene from these organisms for taxonomic purposes. The 16S rRNA gene sequence comparison is well established and has been used successfully to discern the relationship between some closely related and uncharacterized isolates (6). In the present study, analysis of 16S rRNA gene sequence data for *H. cinaedi* and *Flexispira rappini* revealed that this approach has limitations for species-level identification of helicobacters, confirming previously published data (11, 14). Although optimal identification strategies for closely related *Helicobacter* spp. have not been established, a combination of 16S rRNA gene se-

quence analysis and restricted biochemical characterization often does not suffice to identify helicobacters.

Interestingly, all immunocompetent patients and neonates with *H. cinaedi* disease had been in contact with animals. Helicobacters also colonize the gastrointestinal tracts of many animals, including domestic species, such as cats, dogs, pigs, and poultry. Asymptomatic colonization with *H. cinaedi* has also been found in a wide range of animals, for example, rats, hamsters, dogs, cats, foxes, poultry, wild birds, and monkeys. Zenner has proposed that hamsters serve as a reservoir species for zoonotic infection of humans by *H. cinaedi* (18). Al-Soud et al. developed and evaluated a PCR-denaturing gradient gel electrophoresis technique for detection and identification of different *Helicobacter* species (2). Application of the PCR-denaturing gradient gel electrophoresis method to DNA extracted from feces of zoo animals revealed that baboons and red pandas also are colonized by *H. cinaedi*. Although we do not have any definite data about whether the patients with *H. cinaedi* bacteremia had any close contact with animals, it may be possible that the *H. cinaedi* originated with their domestic animals.

Two of the three patients in Toranomon Hospital have the same clinical problem, such as receiving dialysis for chronic renal failure. Interestingly, the first case of *H. cinaedi* bacteremia in Japan involved a patient who also had been receiving dialysis (10). Therefore, we think one of the risk factors for *H. cinaedi* bacteremia may be renal failure and receiving dialysis. We think it is doubtful that the source of infection exists in some of the common services, especially in the dialysis unit, because the period between these two episodes was about 4 months. Furthermore, the dialysis machines are properly maintained and disinfected, and contamination of the system had not been detected by routine examination.

Some clinical reports documented that immunocompromised patients with *H. cinaedi* bacteremia usually require multiple or prolonged courses of antibiotics prior to the resolution of their infections. However, the courses of our patient's illness were not prolonged, and they showed good response to the antimicrobial treatment. One of the reasons for this discrepancy may be the difference in the levels of immunosuppression of the patients. Another reason may be the different choices of antimicrobial agents for the treatment of sepsis. No clear guidelines are available in the literature concerning the choice of antibiotic therapy. Erythromycin is a first-line agent for treatment of these fastidious organisms, but erythromycin-resistant *H. cinaedi* has been identified (7). Ciprofloxacin is an alternative agent for *H. cinaedi* infections, but there are some reports of recurrent disease after fluoroquinolone treatment (3, 4, 8, 15), suggesting that fluoroquinolones alone may not completely eradicate *H. cinaedi* in immunocompromised patients. The results of an in vitro susceptibility test in a case report showed that *H. cinaedi* is susceptible to imipenem (16). In our study, most of the patients with *H. cinaedi* bacteremia were treated with β-lactams, including expanded- and broad-spectrum cephems and carbapenems. Therefore, we recommend using these beta-lactams for the treatment of bacteremia with *H. cinaedi*.

Sequence analysis of *H. cinaedi* isolates showed that three of these isolates had identical 16S RNA sequences. Furthermore, two of these strains were isolated from the same hospital. We next investigated the patients' background information, but they have nothing in common and we could not find any relationship among these cases. Whereas the finding of identical 16S RNA sequences among isolates does not always suggest that they are derived from the same clone, it may be possible that some related isolates are spreading in our country. Therefore, we would like to do further investigation with a greater number of clinical isolates.

In conclusion, our prospective, multicenter analysis finally identified only 6 isolates (0.06%) from 16,743 blood cultures as *H. cinaedi*. All patients with bacteremia with *H. cinaedi* were HIV negative, but most of them were immunocompromised hosts. Although it seems rare in Japan, we should not neglect the possibility of *H. cinaedi* bacteremia.

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