Multi-Center Study to Evaluate Blood Stream Infection with *Helicobacter cinaedi* in Japan.

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Abstract

*Helicobacter cinaedi* has been recognized as an important human pathogen which causes bloodstream infections. Although the first case of bacteremia of 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, multi-center analysis in 13 hospitals during 6 months in Tokyo, Japan. Among positive blood cultures from October 1, 2003 to March 31, 2004, the 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 rDNA 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 containing coagulase-negative staphylococci. Among 9 isolates suspected to be *Helicobacter* species, 6 isolates were finally identified as *H. cinaedi*. The positive rate of *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 HIV infection, but many of them had complication either with 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.
Introduction

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 cause enteric or bloodstream infections. *H. cinaedi* was commonly isolated from homosexual men infected with human immunodeficiency virus. Later, it was also isolated from patients with 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 multi-center study and describe the diagnostic problems in identifying the causative organism.
Materials and methods

Surveillance.

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

Blood cultures 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, tested for urease activity by a selective rapid urea test. Dihydrogen sulfide production,
γ-glutamyltransferase activity, hippurate hydrolysis, and nitrate reduction were determined with the Campy identification system (bioMerieux Vitek, Tokyo, Japan). Some currently available biochemical tests were 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 *Campylobacter* suspected isolates or *Helicobacter* suspected species for further identification.

Patients

Patients with *H. cinaedi* bacteremia were reviewed to determine the clinical background, the source of infection and 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 (CTAB) as described previously (Wilson, 1987). 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 *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, 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, in 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 with the 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 (UPGMA) with Genetyx-win software (version 5.0.4).

Results

Positive rate of blood cultures.
Total 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 (CNS). The positive 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, 9 isolates were suspected to be *Helicobacter* and other related organisms.

Bacterial identification according to biochemical characteristics.

All 9 *Helicobacter* and other related isolates were found to be Gram negative spiral rod, and urease were negative. However, 3 of 9 isolates were suspected to be *Campylobacter* spp. according to the positive growth at 25°C, 42°C, and on CCDA agar (Table 2). Other 6 isolates were biochemically more consistent with *Helicobacter* than with *Campylobacter* based on its 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 the organisms based on the 16S rRNA sequences
The identification results of the isolates based on the 16S rRNA sequences are shown in Table 3. The 16S rDNA sequences of each *H. cinaedi* suspected strains No. 5, 9, 11, 12, 14, and 16, and *H. cinaedi* reference strains ADN0413 (GenBank accession no. M88150.2 for strains No. 5, 9, and 11; HECRR16SAE for strains No. 12, 14, and 16) revealed a high level of similarity (98 to 99%). Furthermore, The 16S rDNA sequences of each *Campylobacter* suspected strains No. 4, 13, 15, and 17, and *C. 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 gene (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 6 cases of bacteremia due to *H. cinaedi* during the surveillance study. The patients with *H. cinaedi* bacteremia were detected from only 3 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).

5 Clinical characteristics

The clinical characteristics of the 6 cases are shown in Table 3. All except one (83%) were female, and the median age was 52 years (range, 17 to 71 years).

At the time of the diagnosis of bacteremia, all patients were hospitalized. Underlying diseases of the patients were as follows: cancer or hematologic disorder was documented for 3 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 hospital stay. All patients recovered after antimicrobial therapy, including second generation cephalosporin and carbapenem.
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 (3). These results suggest that HIV is spreading faster also in Japan. However, the official reports show that HIV infection rates in this country remain still low compared to other countries in the world. 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 then HIV infection.
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, 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 were undergoing chemotherapy with immunosuppressant drugs. We cannot clearly explain the discrepancy between these studies, but these results suggested 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, non-selective media (blood or chocolate agar) incubated in a micro-aerobic (5% O₂) atmosphere at 35°C. It is well documented that the biochemical identification of *Helicobacter* strains based on a limited number of tests is difficult, as these isolates frequently exhibit unusual phenotypic profiles within the same species (15). Therefore, molecular techniques have been used to determine the nucleotide sequence
of the rRNA gene from these organisms for taxonomic purposes. The 16S rDNA 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 *F. rappini* revealed that this approach has limitations for species level identification of helicobacters, confirming previously published data (11, 15). Although optimal identification strategies for closely related *Helicobacter* spp. have not been established, a combination of 16S rDNA sequence analysis and restricted biochemical characterization often does not suffice to identify helicobacters.

Interestingly, all immuno-competent patients and neonates with *H. cinaedi* disease had been in contact with animals. *Helicobacters* also colonize the gastrointestinal tract 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. Gebhart et al. have proposed that hamsters serve as a reservoir species for zoonotic infection of humans by *H. cinaedi*. Al-Soud et al. developed and evaluated a
PCR-denaturing gradient gel electrophoresis (PCR-DGGE) technique for detection and identification of different *Helicobacter* species (1). Application of the PCR-DGGE method to DNA extracted from faeces of zoo animals revealed that baboons and red pandas also colonize *H. cinaedi*. Although we do not have any definite data about the patients with *H. cinaedi* bacteremia had any close contact with animals, it may be possible that the *H. cinaedi* originated from 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 had been also 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 dialysis unit because the period between these two episodes was about 4 months. Furthermore, the dialysis machines are properly maintained and disinfected and the contamination of the system had not been detected by the 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 level of immunosuppression of the patients. Another reason may be the different choice 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 fluoroquinolones treatment (2, 4, 8, 14), 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 the second and third generation cephems and carbapenems. Therefore we recommend using these beta-lactams for the treatment of bacteremia with *H.
Sequence analysis of *H. cinaedi* isolates showed that three of these isolates had identical 16sRNA sequences. Furthermore, two of these strains were isolated from the same hospital. Then we investigated the patients' background information. However, they have nothing in common and we could not find any relationship among these cases. Whereas the isolates with identical 16sRNA sequences do 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 the further investigation with more number of clinical isolates.

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

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References


Figure legends

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences. High degrees of sequence homology among the strains are demonstrated.
Table 1 Results of blood cultures and *H. cinaedi* positive ratio

<table>
<thead>
<tr>
<th>Hospital</th>
<th>No. of total samples</th>
<th>No. of culture positive samples</th>
<th>Culture positive ratio (%)*</th>
<th>No. of H. cinaedi positive samples</th>
<th>H. cinaedi positive ratio (%)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nihon University Itabashi Hospital</td>
<td>2706</td>
<td>430</td>
<td>15.9</td>
<td>0</td>
<td>0.074</td>
</tr>
<tr>
<td>Surugadai Nihon University Hospital</td>
<td>299</td>
<td>59</td>
<td>19.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Showa University Hospital</td>
<td>1417</td>
<td>247</td>
<td>17.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Toho University Omori Medical Center</td>
<td>1200</td>
<td>162</td>
<td>13.5</td>
<td>0</td>
<td>0.083</td>
</tr>
<tr>
<td>Teikyo University Hospital</td>
<td>1084</td>
<td>192</td>
<td>17.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tokyo Medical and Dental University</td>
<td>966</td>
<td>102</td>
<td>10.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tokyo Metropolitan Geriatric Hospital</td>
<td>1154</td>
<td>187</td>
<td>16.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Toranomon Hospital</td>
<td>2860</td>
<td>310</td>
<td>10.8</td>
<td>0</td>
<td>0.104</td>
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<tr>
<td>Mitsui Memorial Hospital</td>
<td>750</td>
<td>124</td>
<td>16.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nakano Sogo Hospital</td>
<td>454</td>
<td>113</td>
<td>24.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Toshiba General Hospital</td>
<td>465</td>
<td>66</td>
<td>14.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Byotai Seiri Laboratory</td>
<td>3089</td>
<td>630</td>
<td>20.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tokyo Koseinenkin Hospital</td>
<td>299</td>
<td>96</td>
<td>32.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>16743</strong></td>
<td><strong>2718</strong></td>
<td><strong>17.7</strong></td>
<td><strong>6</strong></td>
<td><strong>0.036</strong></td>
</tr>
</tbody>
</table>

* Rate of any culture positive samples / total samples (%)
# Rate of *H. cinaedi* positive samples / total samples (%)
<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Institution</th>
<th>Growth at 25°C/42°C</th>
<th>Growth on CCDA agar*</th>
<th>Suspected identification by culture results</th>
<th>16S rRNA sequences</th>
<th>Identification results by the sequence analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Toho University Omori Medical Center</td>
<td>+/+</td>
<td>+</td>
<td>C. fetus</td>
<td>98% · middleF</td>
<td>AF550619.1 C. fetus subsp fetus</td>
</tr>
<tr>
<td>5</td>
<td>Toranomon Hospital</td>
<td>+/-</td>
<td>-</td>
<td>H. cinaedi</td>
<td>98% · middleF</td>
<td>M88150.2 H. cinaedi</td>
</tr>
<tr>
<td>9</td>
<td>Toho University Omori Medical Center</td>
<td>+/-</td>
<td>-</td>
<td>H. cinaedi</td>
<td>99% · middleF</td>
<td>M88150.2 H. cinaedi</td>
</tr>
<tr>
<td>11</td>
<td>Toranomon Hospital</td>
<td>+/-</td>
<td>-</td>
<td>Helicobacter spp</td>
<td>98% · middleF</td>
<td>M88150.2 H. cinaedi</td>
</tr>
<tr>
<td>12</td>
<td>Nihon University Itabashi Hospital</td>
<td>+/-</td>
<td>-</td>
<td>H. cinaedi</td>
<td>99% · 8F + middleF</td>
<td>HECRR16SAE H. cinaedi</td>
</tr>
<tr>
<td>13</td>
<td>Toho University Omori Medical Center</td>
<td>+/-</td>
<td>+</td>
<td>C. fetus</td>
<td>98% · middleF</td>
<td>AF550619.1 C. fetus subsp fetus</td>
</tr>
<tr>
<td>14</td>
<td>Nihon University Itabashi Hospital</td>
<td>+/-</td>
<td>-</td>
<td>Helicobacter spp</td>
<td>99% · 8F + middleF</td>
<td>HECRR16SAE H. cinaedi</td>
</tr>
<tr>
<td>15</td>
<td>Surugadai Nihon University Hospital</td>
<td>+/-</td>
<td>+</td>
<td>C. fetus</td>
<td>99% · middleF</td>
<td>AF550619.1 C. fetus subsp fetus</td>
</tr>
<tr>
<td>16</td>
<td>Toranomon Hospital</td>
<td>+/-</td>
<td>-</td>
<td>Helicobacter spp</td>
<td>98% · middleF</td>
<td>HECRR16SAE H. cinaedi</td>
</tr>
</tbody>
</table>

*Charcoal cefoperazone deoxycholate agar (CCDA) was a specially designed medium for the isolation of Campylobacter spp. and the isolates were incubated microaerobically at 42°C, for 40-48 hours.*
Fig. 1

[GENETYX-WIN : Evolutionary tree]
Date : 2007.4.9
Method: UPGMA

No. 11
0.0007

No. 5
0.0024

No. 14

No. 9

No. 12

No. 16
0.0028

0.0003

0.0000

0.0000

0.0000

0.0000
Table 3 Background data on patients with *H. cinaedi* bacteremia

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Date of sampling</th>
<th>Age</th>
<th>Gender</th>
<th>Underlying disease</th>
<th>Immunosuppressant Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2003/11/8</td>
<td>69</td>
<td>M</td>
<td>Chronic renal failure (on dialysis) Mycosis fungoides</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>2003/11/27</td>
<td>69</td>
<td>F</td>
<td>Primary malignant bone tumor in lower extremity</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>2004/1/6</td>
<td>17</td>
<td>F</td>
<td>Myelodysplastic syndrome</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>2003/12/8</td>
<td>71</td>
<td>F</td>
<td>Endometrial cancer</td>
<td>cisplatin, Adriamycin</td>
</tr>
<tr>
<td>14</td>
<td>2004/2/24</td>
<td>24</td>
<td>F</td>
<td>Threatened premature delivery, Cesarean section</td>
<td>No</td>
</tr>
<tr>
<td>16</td>
<td>2004/3/11</td>
<td>63</td>
<td>F</td>
<td>Chronic renal failure (on dialysis)</td>
<td>No</td>
</tr>
</tbody>
</table>