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MAJOR ARTICLE

Genome Sequencing Verifies Relapsed Infection of *Helicobacter cinaedi*

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Background. Recurrent infections of *Helicobacter cinaedi* are often reported, and long-term antimicrobial treatment is empirically recommended to prevent such infections. However, there have been no studies examining whether recurrent infections are relapses of former infections or reinfections with different clones.

Methods. A 69-year-old woman presented with recurrent *H cinaedi* bacteremia-associated cellulitis after a 51-day interval. We isolated 10 colonies from the blood cultures obtained during each of the 2 episodes and subjected them to whole-genome sequencing (WGS). High-confidence single-nucleotide polymorphisms (SNPs) were identified by an assembly based method. Heterogeneous SNP sites were identified by read mapping. The susceptibility of a representative isolate to 14 antimicrobials was also examined.

Results. Whole-genome sequence analysis revealed only 6 SNP sites among the 20 isolates at the whole-genome level. Based on the 6 SNPs, 5 within-host variants (referred to as genotypes) were identified. All 5 genotypes were detected in the first infection; however, only 2 genotypes were detected in the second infection. Although the *H cinaedi* clone showed a higher minimum inhibitory concentration to fluoroquinolones and macrolides and responsible mutations were identified, none of the 6 SNPs appeared related to additional resistance.

Conclusions. The second infection analyzed here was a relapse of the first infection. A certain level of within-host genomic heterogeneity of the *H cinaedi* clone was already present in the first infection. Our results suggest the importance of longer treatment courses to eradicate *H cinaedi* for preventing the relapse of its infection.

Keywords. *Helicobacter cinaedi*; recurrent infection; relapse; whole-genome sequencing; within-host diversity.

Helicobacter cinaedi, a member of enterohepatic *Helicobacter*, was first isolated from homosexual men and initially recognized as an opportunistic pathogen that causes diseases in immunocompromised patients [1]. However, it is now known that this organism can be isolated from the blood and feces of non-immunocompromised patients [2], indicating that *H cinaedi* infections can occur even in healthy individuals [3, 4]. *Helicobacter cinaedi* infections display a range of clinical manifestations, from bacteremia to cellulitis, enteritis, and meningitis [5–8]. Several outbreaks of nosocomial *H cinaedi* infections have also been reported [9–11]. Another important feature of *H cinaedi* infections is the frequent occurrence of recurrent infections. Araoka et al [12] reported that recurrent infections were observed in 20% of patients with *H cinaedi* infections. However, it is unknown whether these recurrent infections represent reinfections with a different clone or relapses of the initial infection.

In this study, we report the results of the first genome sequencing analysis of isolates obtained from a recurrent *H cinaedi* infection in a patient undergoing long-term dialysis treatment. We analyzed the whole-genome sequences (WGSs) of 20 isolates from the first and second infections, the latter of which occurred 10 weeks after the first. Our results reveal that the first and second infections were caused by the same clone and that the latter was thus a relapse of the initial infection. The presence of within-host genomic diversity of *H cinaedi* was also found.

MATERIALS AND METHODS

Case Description

The patient was a 69-year-old woman who had been undergoing dialysis treatment since the age of 50 due to chronic renal insufficiency. She had additional medical histories, such as gout, interstitial pneumonitis, and angina pectoris, at the ages of 40, 62, and 64, respectively. She was admitted to a hospital in Sapporo City, Hokkaido, where she underwent renal dialysis, with pain

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in both shoulders, buttocks, and lower limbs on December 25, 2015.

After admission, the muscle pain continued, the C-reactive protein (CRP) level was elevated to 6+ (determined by a qualitative test, corresponding to >20 mg/dL; the reference value is <1+), and the white blood cell (WBC) count increased from 8400 to 13 500/μL between the 5th and 11th days after admission, whereas the body temperature remained between 37.0 and 37.5°C. Because cellulitis was suspected for the swelling in both lower limbs, levofloxacin (LVFX), azithromycin (AZM), and cefdinir were administered one after another, but the clinical symptoms did not improve. Hence, the patient was transferred to the Japan Community Healthcare Organization (JCHO) Hokkaido Hospital on January 5, 2016 (see Supplementary Figure 1). At the time of admission, the WBC count was 13 830/μL (neutrophils: 89.8%), and the CRP level was 20.37 mg/dL (reference values: 4000–10 000/μL for WBC, 40.0%–76.0% for neutrophils, 0.30 mg/dL for CRP). Slight swelling in the right lower thigh and both calves, with local redness of skin with unclear margins, and oppressive pain with localized warmth were observed. Because bacterial cellulitis resulting from septicemia was suspected, blood was taken to isolate bacteria, and an intravenous drip of sulbactam and ampicillin (SBT/ABPC) was started. Because a *Helicobacter*-like bacterium was isolated from the blood-culture bottle and identified as *H cinaedi* during the treatment, as described below, this patient was diagnosed as having *H cinaedi* bacteremia-associated cellulitis. On January 18, 2016, the WBC count and CRP level had decreased to normal, and the antimicrobial treatment was changed from intravenous SBT/ABPC to oral tablets and was continued for an additional 2 weeks. The patient was discharged on January 26, 2016, because the symptoms had disappeared.

Fifty-one days after discharge, the woman showed swelling in the right lower limb with light pain along with localized skin redness and had developed a fever of 37.9°C; she was rehospitalized at this hospital on March 17, 2016. The WBC count and CRP level were 10 930/μL and 14.82 mg/dL, respectively (see Supplementary Figure 1). Similar to the first admission, a blood sample was taken for culture, and SBT/ABPC was administered by an intravenous drip for the first 5 days; this treatment was then changed to minocycline (MINO) because antimicrobial susceptibility testing of the *H cinaedi* isolate from the first infection indicated that the isolate showed a lower minimal inhibitory concentration (MIC) to MINO (0.125 μg/mL) and because the possibility of the emergence/selection of an SBT/ABPC-resistant clone by the treatment during the first infection was considered. Minocycline was administered intravenously for 9 days and then by oral tablets for 8 weeks. As *H cinaedi* was isolated from the blood-culture bottle, this patient was diagnosed with recurrent *H cinaedi* bacteremia with cellulitis. The clinical symptoms disappeared, and the patient was discharged on the 21st day after admission. No recurrent infection by *H cinaedi* has developed for 2 years after the second infection.

Microbiological Studies

Blood samples were collected with BACTEC culture bottles and incubated in a BACTEC 9050 blood-culture system (BD Bioscience, Tokyo, Japan) for 5–7 days during the routine diagnosis of bacteremia at the JCHO Hokkaido Hospital. After confirming the growth of spiral-shaped bacteria by direct microscopic examination, an aliquot of each blood culture was inoculated on a Skirrow agar plate [13] and incubated at 37°C for 3–7 days under microaerobic conditions (75% N_2, 10% CO_2, 5% H_2, 10% O_2).

Bacterial identification was made based on the morphological characteristics of colonies and observed in Gram staining, polymerase chain reaction (PCR) detection of the *cdt* gene [14], and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany) [15]. The MICs for 14 antimicrobial agents (see Supplementary Table 1) were determined by the agar dilution method [16]. The type strain of *H cinaedi* (CCUG 18818^T) was obtained from the Culture Collection, University of Göteborg.

Whole-Genome Sequence Analysis

Because we considered a possibility of infections by multiple clones, 10 colonies were randomly picked up from each of the samples obtained in the first and second infections and subjected to WGS analysis. Genomic deoxyribonucleic acid (DNA) of each colony was purified using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Paired-end (PE) multiplex libraries were prepared using a KAPA HyperPlus Kit (KAPA Biosystems, Wilmington, MA) and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA) to obtain 301-base-pair (bp) PE reads. Sequence reads were trimmed using Trimmomatic [17] and assembled using Platanus [18] to obtain draft sequences (see Supplementary Table 2). Contig sequences (≥1 kb) from each isolate were aligned to the draft sequence of isolate F04, which was used as the reference sequence in this study, using NUCmer [19] with a ≥99% identity threshold. Short alignments (≤2 kb) were filtered out to exclude sequences potentially derived from mobile genetic elements. To obtain only high-confidence SNPs, SNPs were excluded if they were within 100 bases of alignment boundaries or in the immediate vicinity (within a 5-bp distance) of any insertion/deletion. Read mapping to the F04 sequence was performed using Burrows-Wheeler Aligner [20] for each isolate to confirm the SNP sequences. In this analysis, when a minor frequency base supported by more than 10% of the mapped reads was detected, both the major and minor base calls were recorded, and the site was treated as a heterogeneous SNP site. Functional prediction of SNP-containing regions was

Table 1. The Six SNP Sites Detected in 20 *Helicobacter cinaedi* Isolates

SNP sites	Isolates From the First Infection										Isolates From the Second Infection										Mutation Type	Annotation
	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10		
SNP-1	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	NS	tRNA-guanine transglycosylase
SNP-2	T	T	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	S	ATP synthase subunit beta
SNP-3	A	A	A	A	A	A	A	A	A	A/G	A	A	A	A	A	A	A	A/G	A/G	G	I	in a 226-bp intergenic region[a]
SNP-4	T	T	T	T/C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	NS	Short-chain dehydrogenase
SNP-5	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	NS	Flagellar motor protein MotA
SNP-6	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	NS	Bifunctional DNA-directed RNA polymerase subunit beta/beta′
Genotype (ratio of genotypes)	G1	G2	G2	G2/G3 (11:89)	G4	G4	G4	G4	G4	G4/G5 (50:50)	G4	G4	G4	G4	G4	G4	G4	G4/G5 (19:81)	G4/G5 (17:83)	G5		

Abbreviations: bp, base pairs; DNA, deoxyribonucleic acid; I, intergenic; NS, nonsynonymous; RNA, ribonucleic acid; SNP, 6 single-nucleotide polymorphism; S, synonymous.

[a]123-bp upstream of the start codon of the *glnQ* gene encoding the glutamine transport ATP-binding protein and 104-bp downstream of the stop codon of a gene for a hypothetical protein.

performed based on the annotation of the draft genome sequence of isolate F04, which was obtained using Prokka [21].

The raw read sequences and assembled scaffold sequences obtained in this study have been deposited in GenBank/EMBL/DDBJ under the BioProject accession number PRJDB7134. Written informed consent was obtained from the patient for publication of this report. This study was approved by the ethical committee of the hospital.

RESULTS

Bacteriological Diagnosis

Spiral-shaped bacteria were grown in cultures of blood collected during the first and second infections. Colonies formed a thin spreading film, and Gram-negative bacteria with a slightly spiral-shaped and slender morphology were observed by Gram staining. The bacteria were positive in the *H cinaedi*-specific PCR for the *cdt* gene and identified as *H cinaedi* in the MALDI-TOF MS analysis, because their intact-cell mass spectrometry profiles analyzed by the MALDI Biotyper 2.0 software showed a high log score of ≥2.

Whole-Genome Sequence Analysis of *Helicobacter cinaedi* Isolates

We isolated and sequenced 20 colonies from the blood cultures from the first and second infections (isolates F01 to F10 from the first infection, and isolates S01 to S10 from the second infection; see Supplementary Table 2 for their sequencing statuses). Whole-genome sequence comparison of the 20 isolates revealed that there were only a total of 6 SNP sites (SNP-1 to SNP-6) among the 20 isolates (Table 1). The SNP-3 and SNP-4 sites were heterogeneous in 4 isolates (F04, F10, S08, and S09) (Table 1), indicating that these isolates contained cells from 2 colonies. This most likely occurred due to the technical difficulty in isolating a single colony of *H cinaedi,* which usually forms film-like colonies. Based on the 6 SNPs, 5 within-host variants (referred to as genotypes G1 to G5) were identified. The 4 heterogeneous isolates had either 2 of the 5 genotypes (G2 and G3 in isolate F04; G4 and G5 in the other 3 isolates). It is interesting to note that all 5 genotypes were detected in the first infection (Figure 1), suggesting that a certain level of within-host genomic diversity was present during the first infection. In the second infection, only 2 genotypes were detected. These findings indicate that the first and second infections were caused by the same clone; thus, the second infection was a relapse of the first infection, and 2 genotypes (G4 and G5) became dominant in the second infection.

Antimicrobial Susceptibilities of the *Helicobacter cinaedi* Clone

Minimal inhibitory concentration measurement of the representative isolate (F04 from the first infection) against 14 antimicrobials (see Supplementary Table 1) revealed that this isolate exhibited a higher MIC to fluoroquinolones ([FQs] ciprofloxacin [64 μg/mL] and LVFX [64 μg/mL]) and macrolides

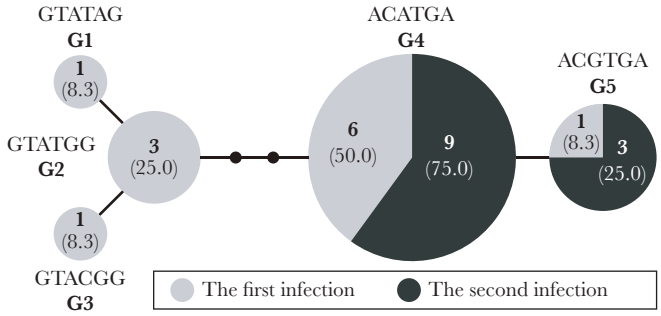

Figure 1. Genetic relationship of the 20 *Helicobacter cinaedi* isolates from the first and second infections. The genetic relationship of the 20 *H cinaedi* isolates, which was inferred based on the 6 single-nucleotide polymorphisms (SNPs) identified by whole-genome sequence (WGS) analysis, is shown. Six genotypes (G1–G6) identified among the 20 isolates are indicated as circles with the concatenate sequences at 6 SNP sites. Two small black circles represent hypothetical intermediates; thus, a distance between 2 neighboring circles represents 1-SNP distance at the WGS level. The size of each circle for the 6 genotypes is in accordance with the number of isolates belonging to each genotype, and the number of isolates from the first and second infections (gray and black backgrounds, respectively) is also indicated in the circles. The number in parentheses indicates the proportion (%) of the isolate in each infection. Note that the total number of isolates for this calculation is 12 for each infection. This is because 2 genotypes were identified in 4 isolates due to the presence of heterogeneous SNP sites (see the main text), and the 2 genotypes were counted as 2 different isolates here.

([MLs] erythromycin [>64 μg/mL] and clarithromycin [64 μg/mL]). In the WGS of isolate F04, we identified 2 mutations in the *gyrAB* gene (T84I for GyrA and D423N for GyrB) and a mutation in the 23S ribosomal ribonucleic acid (RNA) gene (A2059G), which account for the FQ and ML resistance of this isolate. The failure of the treatment in the hospital in which the patient was admitted before being transferred to this hospital was probably due to the FQ and ML resistance because LVFX and AZM were used therein. Notably, all 20 isolates contained these mutations, and none of the above-mentioned 6 SNPs were apparently related to antimicrobial resistance (Table 1).

DISCUSSION

Recurrence is one of the characteristic features of *H cinaedi* infection [12], but there have been no studies examining whether these recurrent infections are relapses of former infections or reinfections with different clones. In this study, we performed WGS analysis of multiple isolates from the initial and recurrent infections. Our results clearly indicate that the second infection was a relapse of the first infection, not a reinfection, suggesting that there was incomplete eradication of the organism colonized somewhere in the host. This analysis also revealed that a certain level of within-host heterogeneity of the *H cinaedi* clone was already present in the first infection. Although *H cinaedi* can be a member of normal microbiota and its infection can occur via translocation from intestinal tracts [2], it is unknown where the observed genetic diversity was generated—in the patient's intestines or some other sites—due to the lack of analyses of *H cinaedi* isolates from fecal samples and of the patient's intestinal microbiota, a limitation of the current study. This patient has developed no *H cinaedi* infection for at least 2 years after the second infection, which might indicate that *H cinaedi* was effectively eradicated by the treatment of the second infection (ie, the prolonged administration of MINO for more than 8 weeks). However, again, this cannot be verified due to the lack of data from the patient's fecal samples. The importance of *H cinaedi* eradication from patients' intestinal microbiota in preventing the relapse of infection needs to be elucidated in the future. Regarding the observed within-host heterogeneity of the *H cinaedi* clone, the selective expansion of 2 genotypes (G4 and G5) is intriguing. One potential mechanism may be selection by antimicrobials. However, none of the 6 SNPs appeared to be related to resistance to the 14 antimicrobials examined, including ABPC used for the treatment of the first infection (Table 1). Three of the 6 SNPs (SNP-1, SNP-2, and SNP-6) were specific to G4 and G5. Two of these 3 SNPs cause nonsynonymous substitutions in the genes for the tRNA-guanine transglycosylase and the RNA polymerase subunits beta/beta', respectively. It is unknown whether these SNPs conferred some increased fitness to these 2 genotypes. However, our current results indicate that the presence of a certain level of within-host heterogeneity should be considered when *H cinaedi* isolates are analyzed.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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