Identification of the Major Antigenic Protein of *Helicobacter cinaedi* and Its Immunogenicity in Humans with *H. cinaedi* Infections

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*Helicobacter cinaedi* infection is now recognized as an increasingly important emerging disease. Its pathogenesis and epidemiological features are not fully understood, however. Here, we investigated the antigenic protein of *H. cinaedi* and the immunological response to it in *H. cinaedi*-infected patients. We constructed a genomic library of *H. cinaedi* from an *H. cinaedi* clinical isolate, and various *H. cinaedi* recombinant proteins were expressed. We identified the 30-kDa protein, encoded in an 822-bp *H. cinaedi* genome, as a major antigen, which was specifically recognized by serum from an *H. cinaedi*-immunized rabbit and *H. cinaedi*-infected patients. The gene encoding this 30-kDa antigen had high sequence similarity with genes encoding putative membrane proteins of bacteria. To evaluate whether the 30-kDa protein can be applied in serological testing for *H. cinaedi* infections, the recombinant protein was expressed in *Escherichia coli* as a His-tagged fusion protein and purified by Ni2+ affinity chromatography. Western blot analysis revealed strong immunoreactivity of the 31-kDa fusion protein with serum antibody from patients infected with *H. cinaedi*, but such an immunoreaction was absent or was very weak with uninfected control serum. An enzyme-linked immunosorbent assay using this *H. cinaedi* major antigen showed significantly high antibody titers for *H. cinaedi*-infected subjects compared with those of various control groups. We therefore conclude that the 30-kDa putative membrane protein is a major antigen of *H. cinaedi* and is useful for immunological and serological testing for clinical diagnosis and for further epidemiological study of *H. cinaedi* infection in humans.

*Helicobacter cinaedi*, which is classified as an enterohepatic *Helicobacter* species, causes bacteremia complicated by cellulitis or arthritis in humans (1, 12–14, 20, 21, 26–28). Although *H. cinaedi* reportedly affects mostly immunocompromised patients (12, 17, 18, 25, 32), we recently observed a series of 11 cases of *H. cinaedi* bacteremia and cellulitis that occurred after orthopedic surgery in a hospital setting in apparently immunocompetent adults (30), and an immunocompetent patient of *H. cinaedi* myopericarditis was reported recently (15). *H. cinaedi* is normal intestinal flora of hamsters (7), and thus zoonotic (hamster-to-human) transmission has been suggested for some cases of neonatal infection (19). The clinical and epidemiological characteristics of *H. cinaedi* infection are not fully clarified, however. For example, although our recent report (13) suggested that an outbreak of *H. cinaedi* infection may have been caused by direct person-to-person spread, a question about the current level of *H. cinaedi* infection in the human community in general remained. Because of the fastidious nature of this organism, which we discovered during bacterial isolation and identification, optimizing culture and isolation methods is a prerequisite to better understanding of the epidemiology and clinical significance of this infection. Blood culture methods utilized in the clinical microbiology laboratory, including microaerobic culture using commercial gas generator envelopes, seem to overlook an appreciable number of *H. cinaedi* infections. In fact, we have had experience with several cases in which this bacterial species was not isolated but the infection was highly suspected because of marked antibody responses as assessed by an enzyme-linked immunosorbent assay (ELISA) using whole-cell antigens from *H. cinaedi* (13).

In this context, the development of immunological assays to analyze the specific immune responses of hosts infected with *H. cinaedi* is important. For example, serological tests such as ELISA, which can be applied exclusively to screening for these infections, are needed and *H. cinaedi* antigens related to immunological responses of *H. cinaedi*-infected hosts should be identified. Several *H. cinaedi* antigens were previously detected via Western blotting with serum obtained from *H. cinaedi*-infected patients, but these antigens did not show consistent antibody response profiles (5). We thus first examined the production of immunoreactive bands that reacted specifically with serum samples obtained from...
Helicobacter cinaedi-infected patients, and we identified, among several immunogenic proteins, a 30-kDa protein with the greatest immunoreactivity. Subsequently, we constructed the H. cinaedi genomic library, which allowed screening for genes encoding the major antigenic proteins, with use of serum specimens from an H. cinaedi-immunized rabbit and H. cinaedi-infected patients. In this study, we identified via rigorous methods the 30-kDa putative membrane protein as a major antigen and reported the human antibody responses to this antigen during H. cinaedi infection.

MATERIALS AND METHODS

Bacteria. As the standard strain for the present genomic and protein analyses, we used a clinical isolate of H. cinaedi (gene accession number of the strain, AB275324 [13]), which we obtained during an outbreak of H. cinaedi bacteremia and cellulitis that we recently described (13). We cultured the isolate via routine methods with Campylobacter agar (Becton Dickinson, Franklin Lakes, NJ) containing 10% sheep blood and incubation at 37°C for 3 to 5 days under microaerobic conditions (CampyPak microaerophilic system; Becton Dickinson) with high humidity. For Western blot analysis of major antigens from H. cinaedi-infected cultures (CampyPak microaerophilic system; Becton Dickinson) with high humidity. For Western blot analysis of major antigens from H. cinaedi-infected cultures, the bacteria were washed, suspended in 10 mM phosphate-buffered saline (PBS) (pH 7.4), and sonicated to yield the H. cinaedi whole-cell lysate as described elsewhere (13). The rabbit was immunized subcutaneously with the lysate, 500 μg of protein in PBS with Freund’s complete adjuvant, followed by two booster doses of 50 μg of lysate protein plus Freund’s incomplete adjuvant at a 2-week interval. Six weeks after the initial immunization, blood was collected to obtain the immune serum. Similarly, antibody was developed for recombinant H. cinaedi protein by means of subcutaneous injection of 480 μg of recombinant protein with Freund’s complete adjuvant and then three booster doses of 480 μg of protein each plus Freund’s incomplete adjuvant.

Serum obtained from patients and other control subjects. Samples of immune serum were collected from eight apparently immunocompetent patients (seven women and one man; ages ranged from 58 to 79 years, with an average of 69.6 years) with bacteremia and cellulitis caused by H. cinaedi who were admitted to Kumamoto Orthopedic Hospital, as we reported previously (13). Details of the clinical characteristics of these patients are reported in our previous paper: patients 1 to 8 (lanes 1 to 8) in Fig. 1A correspond to cases 1, 3, 5, 6, 7, 9, 10, and 11, respectively, documented in Table 1 of our previous paper (13). Another set of serum samples, which were obtained from one patient at various time points after the onset of fever due to H. cinaedi infection in the Kumamoto Orthopedic Hospital, was used for the time profile analysis of anti-H. cinaedi antibody production during infection. This patient (an 82-year-old woman without apparent underlying immunocompromising conditions [patient 5 in Fig. 8 below]) was
developed fever, with a positive blood culture of *H. cinaedi*, at 7 days after surgery for pyogenic spondylitis (for which an etiologic agent could not be identified) and was readily improved by the antibiotic treatment. In addition to the patient sera just mentioned, sera from four patients in the same hospital (all women of ages ranging from 71 to 77 years, with an average of 75.0 years [patients 1 to 4 in Fig. 8 below]) for whom we observed typical clinical symptoms for *H. cinaedi* infections such as cellulitis and fever without immunocompromising diseases were examined for their immune response to *H. cinaedi*. Similar to other *H. cinaedi*-infected patients, all four subjects had orthopedic operations for osteoarthritis (three patients) and bone fracture (one patient) before the onset of clinical symptoms suspected for *H. cinaedi* infection and responded well to the antibiotic chemotherapy. For control subjects, serum samples were obtained from various groups, including 9 age- and sex-matched patients (5 women and 1 man; ages ranged from 57 to 81 years, with an average of 70.6 years) at Kumamoto Orthopedic Hospital who had no apparent *H. cinaedi* infection, 10 *H. pylori*-infected subjects (2 women and 8 men; ages ranged from 37 to 69 years, with an average of 48.2 years) at Oita University Hospital, 5 infants younger than 1 year of age (Oita University Hospital), and 16 healthy volunteers (13 men and 3 women; ages ranged from 21 to 40 years, with an average of 27.4 years) working in the Department of Microbiology at Kumamoto University.

**Ethical compliance.** Procedure for obtaining human serum samples used 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.

**Construction of *H. cinaedi* genomic library.** Genomic DNA was extracted from cultured bacteria by using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Genomic DNA was partially digested with NotI (Takara Shiga, Japan) and DNA was ligated on a agarose gel (Invitrogen Corporation, Carlsbad, CA), and fragments ranging from 4 to 12 kbp were purified with the Qiagen II gel extraction kit (Qiagen). Partially digested genomic DNA was then ligated into the HindIII site of the pBluescript SK (+) vector (Stratagene Inc., La Jolla, CA). The ligation mixture was used to transform *Escherichia coli* ElectroMax DH10B T1 phage-resistant cells (Invitrogen) by electroporation, and then transformed bacteria were plated on Luria-Bertani (LB) agar containing 100 μg/mL ampicillin and 2.5 μg/mL chloramphenicol to get a stable clone. After the colony was confirmed to contain the insert by PCR, the plasmid, pET3d-10, was isolated by using the QIAprep spin miniprep kit (Qiagen). Plasmids from positive clones were isolated by using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Genomic DNA was digested from cultured bacteria by using the DNeasy tissue kit (Qiagen) and its related bacterial species or *H. cinaedi* (0.6 μg/lane) was subjected to sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After SDS-PAGE, proteins were electroblotted onto Immobilon-P membrane (Millipore, Bedford, MA) and then incubated with 5% nonfat dry milk. The membranes were then incubated with various serum samples (diluted from 1:1,000 to 1:625,000 in T-TBS–skim milk) and an antibody: sera were anti-*H. cinaedi* rabbit serum and, as a control, its preimmune serum, with human serum from different groups, including *H. cinaedi*-infected patients, and the antibody was anti-MAP30Hc antibody. The immunoreacted membranes were washed three times with T-TBS and then were incubated further with HRP-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology) diluted 1:5,000 in T-TBS–skim milk. Immunoreactive protein bands were visualized by using an ECL Plus detection system (GE Healthcare) with a luminescent image analyzer (LAS1000 UV Mini; Fujifilm, Tokyo, Japan). In some experiments, to compare the immunoreactivities of protein bands under a consistent immunoblotting condition throughout the study, a membrane after trans-fer of the same protein preparation (e.g., bacterial cell lysate and recombinant protein) was cut into respective membrane strips for each lane of the electrophoresis gel, followed by incubation with different serum samples. After further reaction with the HRP-labeled secondary antibody, the membrane strips were sorted again into each group of *H. cinaedi*-infected and other control sera and were then subjected to the image analyzer to visualize the immunoreactive bands on the same membrane by the same condition. Fujifilm Multi Gauge V3.0 image analysis software served for analysis of band intensity on Western blots. For the reprobing study, each membrane was incubated in a WB stripping solution (Nacalai Tesque, Inc., Kyoto, Japan) for 15 min at room temperature, followed by Western blotting as just described.

**Immunoprecipitation.** *H. cinaedi* whole-cell lysate was prepared by sonicating bacterial cells in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing a protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 12,000 × *g* for 10 min, lysate supernatant was pretreated with protein A/G PLUS-agarose (Santa Cruz Biotechnology) and then incubated with 4°C overnight to eliminate nonspecific binding to the agarose gel. Then, the lysate supernatant (1 mL; 65 μg/mL) was incubated with 5 μg of rabbit normal IgG (Santa Cruz Biotechnology) as a control or with 5 μg of anti- His-MAP30Hc, antibody for 5 h at 4°C, and immune complexes were precipitated by the addition of protein A/G PLUS-agarose for 2 h. The anti-His-MAP30Hc antibody was purified by use of Sepharose CL-4B (GE Healthcare) chromatography and His-MAP30Hc affinity column (HiTrap NHS-activated HP column; GE Healthcare) chromatography. Nonspecific binding was removed from the immunoprecipitated protein A/G PLUS-agarose by four washes with lysis buffer. The immunoprecipitated proteins that were isolated were then analyzed via SDS-PAGE and Western blotting with various serum samples obtained from patients.

**ELISA for determination of the immune response to *H. cinaedi*.** Recombinant His-MAP30Hc was used as an *H. cinaedi* antigen for ELISA. Each well of a 96-well microtiter plate was coated with 100 μL of His-MAP30Hc (0.2 μg of protein/well) in 0.1 M carbonate buffer (pH 9.6), blocked with 1% bovine serum of the same protein preparation (e.g., bacterial cell lysate and recombinant protein) was cut into respective membrane strips for each lane of the electrophoresis gel, followed by incubation with different serum samples. After further reaction with the HRP-labeled secondary antibody, the membrane strips were sorted again into each group of *H. cinaedi*-infected and other control sera and were then subjected to the image analyzer to visualize the immunoreactive bands on the same membrane by the same condition. Fujifilm Multi Gauge V3.0 image analysis software served for analysis of band intensity on Western blots. For the reprobing study, each membrane was incubated in a WB stripping solution (Nacalai Tesque, Inc., Kyoto, Japan) for 15 min at room temperature, followed by Western blotting as just described.

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controls without apparent

H. cinaedi whole-cell lysates (left; 1.3 μg of protein) were electrophoresed and reacted with anti-H. cinaedi rabbit serum (1:1,000 dilution). Molecular masses are given at the right in kilodaltons. (B) Densitometric analysis of the intensity of four immunoreactive bands shown in panel A. The 30-kDa protein (P30) was most dominant. P60, P65, and P90 indicate: 60-kDa, 65-kDa, and 90-kDa proteins, respectively.

FIG. 2. Western blotting assessment of immunoreactivity of H. cinaedi proteins. (A) Serially diluted (dilution, 1-, 5-, 25-, and 125-fold) H. cinaedi-infected and infection-suspected groups, and four different control groups, i.e., H. pylori-infected subjects, age- and sex-matched controls without apparent H. cinaedi infection, infant subjects younger than 1 year old, and volunteers, were compared.

Statistical analysis. Statistical significance between groups was determined via the two-tailed paired Student t test. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession number. The sequence data for MAP30Hc gene determined in this work were deposited in the DDBJ/GenBank/EMBL databases under accession number AB365645.

RESULTS

Antibody responses to H. cinaedi whole-cell antigens in patients with bacteremia. Production of antibodies against H. cinaedi during infections was examined by use of Western blotting. As illustrated in Fig. 1A, among eight patients, five had sera showing a marked immunoreaction with the 30-kDa antigen (lanes 1, 2, 4, 5, and 6), while three showed a moderate or weak reaction (lanes 3, 7, and 8). The times of serum sampling are 249 days, 226 days, 365 days, 120 days, 47 days, 20 days, 18 days, and 7 days after clinical onsets for each patient (lanes 1 to 8, respectively). The intensity of immunoreactive bands therefore seems to correlate with the timing of serum sampling for each patient. For example, much strong immunoreaction was observed with lanes 4 to 6, in which the serum was obtained around a few months after infection. Relatively strong immunoreactive bands with higher molecular masses, ranging from 50 to 100 kDa, were also found. The antibody response for these large antigens appeared to vary among subjects, so a consistent trend in the immune response was not observed. An important finding was the similarity of the immunoreactive pattern for the anti-H. cinaedi rabbit serum (leftmost Western blotting lane in Fig. 1A) and the most prominent reaction (lane 5 in Fig. 1A) to the 30-kDa antigen of the cell lysate proteins.

However, control serum from human subjects (the H. pylori infected, the age and sex matched, and volunteers in the laboratory) showed no appreciable or very weak immunoreactivity with the 30-kDa antigen; weak to moderate reaction was observed for 3 of 33 control subjects (Fig. 1B to D). To verify that the 30-kDa protein is indeed a dominant antigen associated with these bacterial cells, Western blotting was performed with whole-cell lysates serially diluted with anti-H. cinaedi rabbit serum (Fig. 2A) and with anti-H. cinaedi rabbit serum serially diluted against a certain amount of cell lysates (data not shown). Densitometric analysis of immunoreactive protein bands on Western blots clearly indicated that this 30-kDa protein produced the strongest immune response of the proteins (Fig. 2B). We thus focused our work on identification and characterization of the 30-kDa H. cinaedi major antigenic protein.

Identification of antigenic proteins of H. cinaedi. Several clones encoding immunoreactive proteins were isolated via immunoscreening of colonies of E. coli transformants expressing H. cinaedi genomic DNA with use of anti-H. cinaedi rabbit serum. These clones were shown to express H. cinaedi proteins of different sizes, i.e., of 29, 30, 45, 50, and 80 kDa (Fig. 3). Almost the same immunoreactive profiles were observed with serum from an H. cinaedi-infected patient (Fig. 3). Clone 9-5, which contained the corresponding plasmid p9-5, produced a protein with the same electrophoretic mobility as MAP30Hc.
and showed strong immunoreactivity with both rabbit and human anti-H. cinaedi serum samples, as evidenced by Western blotting (Fig. 3). A protein produced by clone 9-5 was almost the same size as MAP30Hc and reacted strongly with both H. cinaedi-immunized rabbit serum and H. cinaedi-infected patient serum. Vector indicates a negative control E. coli clone that had an empty vector. Molecular masses are given at the right in kilodaltons.

Sequencing and characterization of the MAP30Hc gene. The DNA sequence of clone 9-5 was determined, and ORFs therein were predicted. One of the ORFs, which encodes the approximately 30-kDa protein, was presumed to be the MAP30Hc gene. The MAP30Hc gene (GenBank accession no. AB365645) had an ORF of 822 nucleotides, a putative ribosome-binding site (24), a TAA stop codon, and a G+C content of 40%. The ORF showed an identity of 74% in the nucleotide sequence with an ORF of the H. hepaticus gene encoding a 29-kDa conserved hypothetical protein (GenBank accession no. AE017125). The predicted translated protein of the MAP30Hc ORF contained 273 amino acids and had relatively high homology with two other bacterial proteins, according to the NCBI BLAST search (Fig. 4). For example, as just mentioned for the nucleotide sequence homology, the predicted MAP30Hc protein had an identity of 77% with a 29-kDa conserved hypothetical protein of H. hepaticus (GenBank accession no. AAP77310, named 0713Hh). It also had a 57% identity with a 30-kDa outer membrane protein of H. pylori (GenBank accession no. AAD08604, named 1564Hp). It is interesting that the NCBI conserved domain search showed that the MAP30Hc domain was similar to the domain related to ABC-type metal ion transport systems that has wide distribution among bacteria.

In fact, the N-terminal 18-amino-acid peptide fragment of MAP30Hc was presumed to be a probable membrane lipoprotein signal sequence on the basis of the database LipoP, used to predict lipoprotein signal peptides in gram-negative bacteria (http://www.cbs.dtu.dk/services/LipoP/). Searching the same database suggested that the amino acids at positions 17 and 18 serve as a putative cleavage site for a specific lipoprotein signal peptidase, signal peptidase II (9, 23), and that the Cys at position 18 may be a site of attachment to a membrane lipid (9). It is thus highly plausible that MAP30Hc is a membrane protein related to the bacterial ABC-type metal ion transport system. As described above, Fig. 4 compares predicted amino acid sequences of the MAP30Hc protein with H. hepaticus protein 0713Hh and H. pylori protein 1564Hp, illustrating a relatively high degree of homology.

Characterization of recombinant MAP30Hc as a major antigen of H. cinaedi. The MAP30Hc ORF was amplified by PCR, and amplicons were directionally cloned in frame into vector pET3d to obtain plasmid pET3d-10His-HcMAP30 for use in the recombinant protein expression system. His-MAP30Hc recombinant protein expressed in and purified from E. coli served as an antigen for analysis of the immunoreaction of antibodies in rabbit and human anti-H. cinaedi sera (Fig. 5A)
and B). The 31-kDa His-MAP30Hc fusion protein strongly reacted not only with serum from an H. cinaedi-immunized rabbit but also with serum from an H. cinaedi-infected patient. A few immunoreactive bands of relatively high molecular mass were found for serum samples from rabbits immunized and patients infected with H. cinaedi that were reacted with whole-cell lysates of H. pylori, H. hepaticus, and C. jejuni (Fig. 5A and B). However, protein bands similar to those for MAP30Hc were not observed for other bacteria tested, except for the weak signal of an approximately 29-kDa protein detected for H. hepaticus.

A slight difference in electrophoretic mobilities of MAP30Hc immunoreactive bands was noted for the recombinant His-MAP30Hc and the H. cinaedi whole-cell lysate, which was most likely due to the His repeat peptides added to the fusion protein. That the recombinant MAP30 Hc protein and the nat-

![FIG. 5. Western blot analysis, after electrophoresis, showing reactivity of sera from an H. cinaedi-immunized rabbit (1:15,000) (A), an H. cinaedi-infected patient (1:1,500) (B), and a His-MAP30Hc-immunized rabbit (1:15,000) (C) to His-MAP30Hc (0.6 μg) and whole-cell lysates (6.5 μg) of four bacterial species (H. cinaedi, H. pylori, H. hepaticus, and C. jejuni). A membrane blotted in panel A was stripped and then used for blotting in panel C. Black arrowheads and open arrowheads in panels A to C indicate MAP30Hc and His-MAP30Hc, respectively. SDS-PAGE of recombinant His-MAP30Hc (Coomassie brilliant blue [CBB] staining) is illustrated at the left of panel A. (D) H. cinaedi whole-cell lysate (6.5 μg) was incubated with anti-His-MAP30Hc antibody or normal rabbit IgG (0.5 μg) for 5 h at 4°C. Proteins recognized by antibodies were precipitated with protein A/G PLUS-agarose beads and then applied to Western blots with H. cinaedi-infected patient serum. A protein immunoprecipitated with anti-His-MAP30Hc antibody reacted with the H. cinaedi-infected patient serum (arrowhead) and was the same size as MAP30Hc in H. cinaedi whole-cell lysate (6.5 μg/lane) (right). Molecular masses are given at the left in kilodaltons. HcMAP30, MAP30 Hc.

![FIG. 6. Western blot analysis of responses to recombinant His-MAP30 Hc in serum from H. cinaedi-infected patients (A) and controls (B to D). Sample (lane) numbers above the image correlate with those in the respective groups in Fig. 1. Each lane contained 0.6 μg of His-MAP30Hc. All serum samples were diluted 1:1,500. Responses for the H. cinaedi-infected patients (A) and controls (B to D) were similar to those for H. cinaedi whole-cell lysate (Fig. 1). With a few exceptions, e.g., lane 1 of the H. pylori-infected group (B), lane 8 of the age- and sex-matched control group (C), and lane 5 of the healthy volunteer group (D), control sera did not react to this protein (only a few representative lanes for control negative reactions are shown in panels B to D). The arrowhead indicates His-MAP30Hc. Molecular masses are given at the left in kilodaltons.](image-url)
FIG. 7. Recombinant His-MAP30Hc-based ELISA results. Serum antibody levels of *H. cinaedi*-infected patients (*n* = 8) were compared with those of control groups: *H. pylori*-infected patients (*n* = 10), controls 1 (age- and sex-matched subjects) (*n* = 9), controls 2 (healthy volunteers) (*n* = 16), and infants (*n* = 5). Numbers for the *H. cinaedi*-infected patients and control groups correlate with the numbers of respective groups in Fig. 1 and 6. Black circles indicate data for individuals in the patient and control groups; open boxes indicate averages, with standard deviations indicated. *, *P* < 0.05.

ELISA titers correlated well with the intensities of immunoreactive bands illustrated in Fig. 1 and 6. More importantly, as shown in Fig. 8, we also examined the time course of antibody responses for four patients (no. 1 to 4) for whom infection was strongly suspected because of typical clinical symptoms of *H. cinaedi* infection but from whom this bacterial species was not isolated. One patient (a 71-year-old woman) showed a drastic elevation of antibody titer, and three patients showed a slow but substantial rise in the response to His-MAP30Hc antigen (Fig. 8). Similarly, the antibody titers of patient 5 (an 82-year-old woman), who was truly infected with *H. cinaedi*, showed a clear increase in antibody production after *H. cinaedi* infection.

DISCUSSION

In our previous report, we suggested that *H. cinaedi* infection may be more widespread in the general world community than previously believed (13). Optimal conditions for isolation of this species from clinical specimens have not been established, and specific conventional approaches such as serological assays are required to better define the pathogenic and epidemiological features of this emerging pathogen.

Our present study revealed that a 30-kDa immunogenic protein is the most potent of various *H. cinaedi* proteins for evoking an immune response in hosts. Flores et al. (5) described several immunogenic *H. cinaedi* antigens to serum from infected patients. The specific host immune response to His-MAP30Hc was examined by determining antibody titers. Figure 7 demonstrates that patients’ antibody titers were significantly higher (*P* < 0.05) than those of all control groups. The levels of

FIG. 8. Time course of serum response in patients with suspected or true *H. cinaedi* infection and analyzed via recombinant His-MAP30Hc-based ELISA. Day 0 indicates the time that cellulitis (patients 1 to 4) and fever (patient 5) were first recognized in the patients. Patients 1 to 4 showed typical symptoms of *H. cinaedi* infection though the bacteria were not isolated from them, while *H. cinaedi* was isolated from the blood of patient 5.
exhibited the strongest reactions. In fact, we found relatively strong immunoreactivity for the 60- and 65-kDa proteins in our Western blot analysis (Fig. 1A and 2). However, because we observed similar bands with controls to some degree, these 60- and 65-kDa proteins may not be the major antigens functioning during *H. cinaedi* infections (Fig. 1B to D). Our dilution experiments with antigen and antisera confirmed that the 30-kDa antigen, identified as MAP30Hc, had the strongest immune response among the different proteins of whole-cell *H. cinaedi* lysates (Fig. 2).

One of eight *H. cinaedi*-infected patients showed very little reaction to the 30-kDa protein (Fig. 1A, lane 7), and two had only a weak reaction (Fig. 1A, lanes 3 and 8), findings that correlated well with results of recombinant MAP30Hc-based Western blotting (Fig. 6A, lanes 3, 7, and 8) and ELISA (Fig. 7). A similar correlation was observed for the immunoreactivity of various control groups between Western blotting (Fig. 1B to D and 6B to D) and ELISA (Fig. 7). The variability in the host immune reactions to *H. cinaedi* antigens is thought to be due to differences in the time courses of infection and antibody development at the time of the serum sampling. In fact, the strongest immune response was observed with the serum obtained a few months after the onset of clinical symptoms of *H. cinaedi* infection, as evidenced by the Western blotting and ELISA data from lanes 4 to 6 (patients 4 to 6) in Fig. 1A, 6A, and 7. Such a time-dependent antibody production might be reasonably understood because of the well-known time profile of the host’s immune response against various pathogens in general. Alternatively, it may reflect distinct individual immune responses to the specific antigen. As just discussed, in *H. cinaedi*-infected patients (seven women and one man), high antibody titers were seen with two women and one man (patients 4 to 6 in Fig. 7). The average age of all patients was 69.6 years, and that of three patients with high titers was 69.7 years. Thus, there is no particular tendency in age or gender for prominent immune response to MAP30Hc. Details of the clinical characteristics of the present patients are described in our previous paper (13), which indicate again that no substantial difference was found in their clinical characteristics among the patients showing varied antibody titers. Similarly, we could not find any clear factors (e.g., age, gender, and clinical background) associated with a high antibody production for MAP30Hc among the control groups tested in our current study.

This 30-kDa protein, which we identified here as a major immunogenic protein (MAP30Hc), has homology with a 29-kDa protein of *H. hepaticus* (0713_H16, GenBank accession no. AAP77310). Although the function of this 29-kDa *H. hepaticus* protein is not clear, database information is available for a similar protein, an *H. pylori* putative outer membrane protein (1564_H16, GenBank accession no. AAD08604). In addition, via an NCBI conserved domain search, we found a similarity to a domain of the ABC-type metal ion transport system, periplasmic component/surface antigen. Thus, it is highly plausible that MAP30Hc is a membrane lipoprotein related to the bacterial ABC-type metal ion transport system.

Western blotting with *H. hepaticus* whole-cell lysates demonstrated that rabbit anti-*H. cinaedi* serum strongly reacted with antigens of high molecular mass (50 to 100 kDa) and weakly reacted with the 29-kDa antigen (Fig. 5A); rabbit anti-His-MAP30Hc antibody showed a moderate reaction to the 29-kDa antigen of *H. hepaticus* (Fig. 5C). These results suggest that MAP30Hc and the 29-kDa protein of *H. hepaticus* are antigenically cross-reactive. More-marked cross-reactions were observed between high-molecular-mass lysate proteins of *H. cinaedi* and those of other related bacteria such as *H. hepaticus*, *H. pylori*, and *C. jejuni* (Fig. 5A and B). Several investigators have suggested a possible association of *H. hepaticus* with human diseases (16, 31, 33). Indirect support for this idea comes from the detection of *H. hepaticus* 16S rRNA gene sequences in DNA extracted from colonoscopy samples obtained from children with inflammatory bowel disease (33) and from the finding that an *H. hepaticus* cell extract-based ELISA showed elevated antibody levels in patients with chronic liver diseases compared with controls (31). However, *H. hepaticus* is generally recognized as a pathogen that causes hepatitis in mice (6) rather than as a human pathogen: no confirmed evidence exists of isolation of *H. hepaticus* from human specimens, which would prove pathogenesis in humans. Moreover, the cross-reactivity between *H. cinaedi* and *H. hepaticus* antigens, as revealed in the present work, suggests that false-positive ELISA results for human infections may have been attributed to *H. hepaticus* but may have actually been caused by *H. cinaedi*. In contrast, human anti-*H. cinaedi* serum produced no measurable immunoreaction for the 29-kDa protein of *H. hepaticus* (Fig. 5B). Thus, the MAP30Hc protein that we identified here may prove useful as a major antigen in serological assays for *H. cinaedi*, with high specificity in screening patients with various infections caused by Helicobacter and related bacteria.

Several studies have reported immunogenic proteins from Helicobacter or Campylobacter species and examined recombinant protein-based ELISAs (2–4, 11, 16, 29). The specificity of these ELISAs seems to be greater than that of ELISAs using whole-cell antigens. Similarly, the His-MAP30Hc-based ELISA used here has high specificity, because the nontspecific ELISA titer obtained for control subjects was much lower than that in the whole-cell protein-based ELISA (13). Although the host antibody response seems to vary depending on the time of serum sampling (for *H. cinaedi*-infected patients, from 7 days to 1 year from the onset of clinical symptoms), the present MAP30Hc-based ELISA revealed a significantly higher antibody titer for *H. cinaedi*-infected patients than for controls (*P* < 0.05) (Fig. 7). The relatively high antibody level detected for a few control subjects (Fig. 7) suggests the presence of subclinical *H. cinaedi* infection in the general population. All these ELISA patterns in *H. cinaedi*-infected and control groups were consistent with profiles of the MAP30Hc immunoreactive band found via Western blotting, as described above (Fig. 1 and 6). We therefore believe that the ELISA using MAP30Hc as an antigen may become a potent serological tool for screening humans for *H. cinaedi* infections.

The time course analysis of antibody responses in the group with suspected *H. cinaedi* infection and its true infection indicated that these patients had different degrees of increase in antibody titers (Fig. 8). One explanation for this variation may be different individual immune responses to specific antigens of this microorganism. Also, a determinant may be whether hosts had been previously immunized by the antigen during subclinical infections, as mentioned earlier for control subjects
with relatively high antibody titers. Nevertheless, for typical clinical cases, *H. cinaedi* infection may be diagnosed by means of this serological procedure that utilizes the ELISA described here and does not involve bacterial culture.

In conclusion, we identified in our current study a 30-kDa *H. cinaedi* putative membrane protein as a major antigen (MAP30Hc) and successfully constructed the expression system of the MAP30Hc. His-tagged fusion recombinant protein. This recombinant protein has great potential for use in serological assays, including Western blotting and ELISA, for clinical diagnosis of *H. cinaedi* infection. More important, the present identification of MAP30Hc not only aids better understanding of the etiology of *H. cinaedi* infections but also provides evidence to warrant further epidemiological study of these infections in various hosts in nature.

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