The pathogenic potential of Helicobacter cinaedi isolated from non-human sources: adherence, invasion and translocation ability in polarized intestinal epithelial Caco-2 cells in vitro

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ABSTRACT. Helicobacter cinaedi infection has been recognized as an increasingly important emerging disease in humans. Infection with H. cinaedi causes bacteremia, cellulitis and enteritis. H. cinaedi has been isolated from non-human sources, including dogs, cats and rodents; however, it remains unclear whether animal strains are pathogenic in humans and as zoonotic pathogens. In this study, H. cinaedi isolates were recovered from a dog and a hamster, and the ability of these isolates to adhere to, invade and translocate across polarized human intestinal epithelial Caco-2 cells was examined in vitro. To better understand the pathogenic potential of animal H. cinaedi isolates, these results were compared with those for a human strain that was isolated from a patient with bacteremia. The animal and human strains adhered to and invaded Caco-2 cells, but to a lesser degree than the C. jejuni 81–176 strain, which was used as a control. The integrity of tight junctions was monitored by measuring transepithelial electrical resistance (TER) with a membrane insert system. The TER values for all H. cinaedi strains did not change during the experimental periods compared with those of the controls; however, translocation of H. cinaedi from the apical side to the basolateral side was confirmed by cultivation and H. cinaedi-specific PCR, suggesting that the H. cinaedi strains translocated by transcellular route. This study demonstrated that H. cinaedi strains of animal origin might have a pathogenic potential in human epithelial cells as observed in a translocation assay in vitro with a human isolate.

KEY WORDS: adherence, Caco-2 cell, Helicobacter cinaedi, invasion, translocation

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Helicobacter cinaedi, first recognized as a Campylobacter-like organism (CLO), is a Gram-negative, spiral-shaped, motile, microaerobic bacterium and classified into enterohepatic Helicobacter species [25]. This organism was first isolated from homosexual men and was initially recognized as a rectal and intestinal pathogen among members of the population [25]. Since its isolation in humans, H. cinaedi has been considered an opportunistic pathogen that causes bacteremia, cellulitis and enteritis in immunocompromised [2, 13, 19], immunocompetent and healthy persons [10, 15, 18]. Because H. cinaedi strains have been isolated from blood in humans [15, 19], the organism may be able to pass through the intestinal barrier. However, the epidemiological features of H. cinaedi, including its infectious sources and routes and pathogenesis, remain unclear.

According to epidemiological surveys, H. cinaedi has been isolated from non-human sources, such as dogs, cats, monkeys, hamsters and other rodents [4–6, 14, 26], which suggests that the organism may be widespread in a wide range of animal species. Therefore, these animals may be reservoirs for human infections [4, 6]. However, the pathogenesis and epidemiological features of H. cinaedi strains of non-human origin are not fully understood. Therefore, we believe that it is important to determine whether animal strains of H. cinaedi have pathogenic potential in humans as a zoonotic disease.

Bacterial infection using cultured mammalian cells in vitro is a useful tool for investigating host epithelial cell-bacterium interactions, because a single population of organisms can be infected under defined conditions. These assays are simple and reproducible and allow for the quantification of both adherent and internalized bacteria. In this study, we compared the adherence, invasion and translocation ability of human and non-human H. cinaedi strains in polarized epithelial Caco-2 cells that were derived from the human intestine in vitro.
MATERIALS AND METHODS

Bacterial strains: Three *H. cinaedi* strains were used in this study and have been previously described [27]. The N73 strain was isolated from the blood of a patient with bacteremia. The T22 and T34 strains were isolated from the feces of a healthy dog and hamster, respectively. The *H. cinaedi* CCUG 18818T strain was obtained from the Culture Collection of the University of Göteborg, Sweden and was used as a positive control for *H. cinaedi*-specific PCR. The *Campylobacter jejuni* strain was originally obtained from a milk-borne outbreak in Minnesota [17], was used as a control, because its adherence to and invasion ability in intestinal epithelial Caco-2 cells has been well characterized [8, 11]. All of the bacterial strains were grown on Brucella agar (Becton, Dickinson and Co., Tokyo, Japan), which contained 5% defibrinated horse blood (Nippon Biotest Laboratories Inc., Tokyo, Japan), at 37°C for 2–3 days under microaerobic conditions (80% N₂, 10% CO₂, 5% O₂, and 5% H₂).

All of the isolates were suspended in Brucella broth (Becton, Dickinson and Co.), which contained 10% (v/v) glycerol (Kanto Kagaku, Tokyo, Japan), and stored at −80°C until testing. Before use in experiments, the bacteria were grown on Brucella agar, which was supplemented with 5% defibrinated horse blood, at 37°C for 2–3 days under microaerobic conditions as previously described.

Cell culture: Caco-2 (human colon carcinoma) cells were obtained from the RIKEN BioResource Centre Cell Bank (Tsukuba, Japan) and were routinely cultured in Eagle’s minimum essential medium (EMEM; Nacalai Tesque, Kyoto, Japan), which was supplemented with 10% fetal bovine serum (FBS; NICHIREI BIOSCIENCES INC., Tokyo, Japan) and contained 50 µg/ml kanamycin (Sigma-Aldrich Japan, Tokyo, Japan), at 37°C under a 5% CO₂ humidified atmosphere.

Adherence and invasion assays: The adherence and invasion assays were performed according to the method by Konkel et al. [16] with several modifications. Briefly, 3 × 10⁵ Caco-2 cells were added to each well of a 24-well microplate (ASAHII GLASS Co., Ltd., Tokyo, Japan), and the cells were incubated at 37°C for 2–3 days under microaerobic conditions as described above. The TER value reached maximal polarity (>330 Ω/cm²) after 7–10 days of incubation, the monolayers were washed three times with PBS. Then, approximately 2–5 × 10⁵ cfu of *H. cinaedi*, suspended in 200 µl of EMEM containing 1% FBS without antibiotics, was inoculated into the apical side of the monolayer. The culture medium in the basolateral side was replaced with 1 ml of EMEM that contained 1% FBS without antibiotics. The TER values were measured at 6, 12 and 24 hr after infection. The TER values for the cell-free control well were subtracted from the obtained values to remove background values. The cell culture inserts that were not inoculated with bacteria were used as a negative control. The assay was tested at least three times in triplicate.

Detection of *H. cinaedi* cells in the basolateral compartment of the cell culture insert: To determine whether the *H. cinaedi* strains translocated across the monolayer of Caco-2 cells on a transmembrane of the cell culture insert, the bacterial cells in the medium of the basolateral side were harvested by centrifugation at 10,000 × g for 10 min after a predetermined inoculation time. The precipitation was resuspended in 100 µl of PBS, and 100 µl of the suspension was inoculated on Brucella agar plates that contained 5% defibrinated horse blood and incubated at 37°C for 2–3 days under microaerobic conditions as described above. The bacterial DNA from 100 µl of the suspension was extracted using the DNasy Blood, and Tissue Kit (Qiagen, Tokyo, Japan) was used according to the manufacturer’s instructions. The PCR amplification of the cdt gene, which encodes cytolethal disintegrating toxin, was used to specifically detect *H. cinaedi* [24]. The partial cdt gene (410 bp) of *H. cinaedi* was amplified with the primers CDT F (5’- CTCGTCGGGATATGGTG -3’) and CDT R (5’- AGAGTTCCCTATCCTGTC -3’), which were designed based on the cdt gene-sequence in *H.
cinaedi (accession number AB275331). PCR was performed in a final reaction volume of 20 µl. Each reaction contained 20 pM of each primer (forward and reverse), 200 µM of each deoxynucleoside triphosphate, 0.5 U of Taq DNA polymerase (Qiagen), 1× PCR buffer and 2 µl of extracted DNA. The thermal cycling conditions included 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The specific amplification of each target gene was confirmed by 2% agarose gel electrophoresis.

Immunization of rabbit for H. cinaedi antibody production: Polyclonal antisera against H. cinaedi were prepared with 2-month-old New Zealand white rabbits. In brief, the rabbits were inoculated intracutaneously with each emulsified mixture of bacterial suspension and Freund’s incomplete adjuvant (Nacalai Tesque) on three different days with 2-week intervals. Two weeks after the third immunization, whole blood was collected from the immunized rabbits. The collected serum was inactivated by incubation at 56°C for 30 min in a water bath and was stored in aliquots at −20°C until use. The protocol was approved by the committee on animal experiment at the University of Miyazaki (acceptance number; 2007-025-6).

Fluorescence microscopy: To observe internalized H. cinaedi in Caco-2 cells after invasion, an immunofluorescence procedure was performed. Trypsinized 1 × 10⁵ Caco-2 cells were placed into each well of a chamber slide (Nalgene) using the fluorescence microscope BIOREVO BZ-9000 (KEYENCE, Osaka, Japan). The presence of H. cinaedi strains in Caco-2 cells, the monolayers were examined were recovered from the basolateral medium by cultivation and PCR during 24 hr observed (Fig. 1C). Regardless of the isolate origin, all of the H. cinaedi strains exhibited invasion potential in Caco-2 cells.

RESULTS

Adherence and invasion in Caco-2 cells: All of the H. cinaedi strains adhered to and invaded Caco-2 cells regardless of their origin (Table 1). However, the number of H. cinaedi that adhered to and invaded Caco-2 cells was lower than that of C. jejuni. The number of adherent and internalized bacterial cells was compared among the tested strains. Strain N73, which was isolated from a patient with bacteremia, adhered to and invaded Caco-2 cells in greater numbers (10⁶ cfu for adhesion and 10⁴ cfu for invasion, respectively) compared with the canine strain T22 (10⁵ cfu for adhesion and 10³ cfu for invasion) and the hamster strain T34 (10⁴ cfu for adhesion and 10 cfu for invasion).

Analysis of epithelial barrier integrity: The ability of H. cinaedi strains to translocate across epithelial cells was examined in polarized Caco-2 cell monolayers using a membrane insert system. TER across polarized monolayers of the Caco-2 cells was not affected by any of the H. cinaedi strains during the experimental period, which was similarly observed for the negative control and C. jejuni (Fig. 1A). Migration of H. cinaedi from the apical side to the basolateral side was confirmed at 6 hr after the inoculation by cultivation and H. cinaedi-specific PCR using the basolateral medium (Fig. 1B and 1C), and all of the H. cinaedi strains examined were recovered from the basolateral medium by cultivation and PCR during 24 hr observed (Fig. 1C).

DISCUSSION

Several researchers reported that direct contact with pets that harbor H. cinaedi may be a possible route of infection in humans [13, 20, 23]. However, data on the pathogenesis and epidemiology of animal H. cinaedi strains, including zoonotic transmission routes between animals and humans, are not fully understood. Despite the high prevalence of H. cinaedi in hamsters [6], intestinal disease does not occur following naturally acquired infection. Therefore, H. cinaedi may behave as a commensal in animal hosts, because clinical and pathological observations have not been reported. Misawa et al. [21] reported that the H. cinaedi strain 94105 was isolated from a 2-month-old female puppy with bloody diarrhea. In this case, the diarrhea episodes occurred repeatedly after antibiotic treatment, and H. cinaedi was isolated from the puppy each time. However, because Campylobacter upsaliensis, Helicobacter bilis and Anaerobiospirillum spp. were simultaneously isolated, it is unclear whether H. cinaedi was the direct cause of the diarrhea. Therefore, the pathogenesis of H. cinaedi that is isolated from animals has been controversial. This lack of knowledge has confounded the prevention and treatment of this infection.

To investigate the host epithelial cell-enteric bacterium
Fig. 1. Transepithelial electrical resistance (TER) in Caco-2 cells infected with *H. cinaedi*. (A) TER was determined at 0, 6, 12 and 24 hr after infection. The values are expressed as the percentage of TER immediately before bacterial inoculation. The values are the mean (n=3) ± standard deviation. One representative, in triplicate, of three experiments is shown. The differences from the control at each observation point were not significant. (B) The presence of *C. jejuni* and *H. cinaedi* that migrated from the apical side to the basolateral side during cultivation on the basolateral medium was expressed as: -, not detected; or +, detected. (C) *H. cinaedi* that migrated from the apical side to the basolateral side was detected using an *H. cinaedi*-specific PCR with DNA that was extracted from the basolateral medium. The CCUG 18818T strain was used as a positive control for *H. cinaedi*-specific PCR.

Table 1. The numbers of viable *H. cinaedi* and *C. jejuni* that adhered to and invaded Caco-2 cells

| Strain | Origin  | The number of viable that inoculated per well in a 24-well plate | The number of viable that adhered to cells per well in a 24-well plate | The number of viable that invaded cells per well in a 24-well plate |
|--------|---------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| N73    | human   | $1.35 \times 10^8$                                            | $^{a}(1.32 \pm 0.29) \times 10^6$                            | $(4.40 \pm 0.14) \times 10^4$                                 |
| T22    | dog     | $4.90 \times 10^7$                                            | $(3.38 \pm 1.37) \times 10^5$                                | $(9.00 \pm 1.44) \times 10^3$                                 |
| T34    | cat     | $5.27 \times 10^7$                                            | $(2.33 \pm 0.81) \times 10^4$                                | $(1.67 \pm 1.15) \times 10$                                   |
| 81–176 | human   | $6.45 \times 10^7$                                            | $(1.59 \pm 0.40) \times 10^6$                                | $(3.88 \pm 0.42) \times 10^5$                                 |

a) The results were expressed as the mean ± the standard deviation for every experiment, which were performed in triplicate.

Fig. 2. Three-dimensional images of Caco-2 cell monolayers infected with *H. cinaedi* at 24 hr post inoculation using a fluorescence microscope (Z-axis scans, XY plane). Cytoskeletal actin is stained red with rhodamine phalloidin. *H. cinaedi* is stained green using an anti-*H. cinaedi* antibody and FITC-conjugated goat anti-rabbit antibody. The scale bars represent 10 µm. (A) The N73 strain of human origin in Caco-2 cells, (B) The T22 strain of canine origin in Caco-2 cells, (C) The T34 strain of hamster origin in Caco-2 cells.
interaction, Caco-2 cells are commonly used as a simple model. Caco-2 cells are derived from transformed human colonic carcinoma cells, which are polarized monolayers that have intercellular tight junctions and defined apical and basolateral structures [9, 12]. This study was conducted to better understand the pathogenesis of H. cinaedi strains that are isolated from animals using this in vitro model. Potential virulence factors that may play a role in the pathogenesis of H. cinaedi include adherence, invasion and translocation across the mucosal epithelial barrier. This study demonstrated that a human H. cinaedi strain and H. cinaedi strains isolated from a dog and hamster exhibited adherence and invasion potential in human epithelial cells in vitro. This finding was similarly observed in the C. jejuni strain 81–176, whose adhesion and invasion potential in Caco-2 cells has been well characterized (Table 1). However, as compared with C. jejuni, which is a causative microorganism of food-borne infections, the number of adhering and invading H. cinaedi cells was low. Therefore, the adherence and invasion ability of H. cinaedi in Caco-2 cells may be lower than that of C. jejuni. In addition, the adherence and invasion ability of the T34 strain, which was isolated from a hamster, was lower than those of canine and human strains. These differences may be attributed to pathogenic abilities, such as motility; the characteristics of the bacterial surface, which depend on the type of species which the strains are derived or the experimental conditions, such as the MOI [15]. Additional studies using more strains under different experimental conditions are needed; however, the finding that animal strains have the same pathogenic potential as a human strain is more important than a comparison of the pathogenic abilities among animal strains.

The possible pathways that H. cinaedi uses to penetrate the intestinal mucosa are considered transcellular and/or paracellular routes [1]. The ability of the human and animal H. cinaedi strains to translocate across the epithelial cell barrier was examined in polarized Caco-2 cell monolayers by measuring TER (Fig. 1). TER is used frequently as an index of tight junction permeability and monolayer integrity. When bacteria use the intercellular route from the apical to basolateral cell surface, the tight junction is disrupted and TER decreases [3]. In this study, none of the H. cinaedi strains were associated with a decrease in TER after 24 hr of infection as seen in C. jejuni 81–176, which can translocate from the apical side to the basolateral side of polarized Caco-2 cells without varying TER value [28], indicating that H. cinaedi did not disrupt the tight junctions of Caco-2 cells. Whereas the bacteria inoculated into the apical side were isolated from basolateral medium 6, 12 and 24 hr after inoculation (Fig. 1B). Furthermore, a partial cdt gene-fragment, which was specific to the H. cinaedi strains, was detected by PCR in the basolateral medium (Fig. 1C). This finding suggests that H. cinaedi uses transcellular translocation and has transcytotic ability. During transcytosis, pathogens invade epithelial cells, migrate across the cytoplasm and egress from the opposite surface. To confirm the internalization of H. cinaedi in Caco-2 cells, the monolayers were infected with the three H. cinaedi strains, and internalization was visualized using immunofluorescent techniques. As expected, all of the H. cinaedi strains were observed in Caco-2 cells regardless of the origin (Fig. 2). These observations suggest that H. cinaedi strains might migrate by transcellular translocation.

This study demonstrated that animal strains have the adhesion and invasion ability as seen in human strains, especially isolates from a dog and a hamster, which are common companion animals. This report is the first on the pathogenesis of H. cinaedi using polarized intestinal epithelial Caco-2 cells in vitro. The in vitro pathogenic potential may explain human clinical cases of bacteremia, cellulitis and septicemia [2, 13, 18, 19]. To date, no reliable epidemiological evidence of the zoonotic potential of H. cinaedi has been published. Therefore, further investigations are needed to determine the epidemiology and infection sources of animal H. cinaedi strains in humans.

The whole genome of H. cinaedi has recently been reported, and several pathogenic genes were present in the genome. HCN_1887 (fibronectin/fibrinogen-binding protein) and ciaB (Campylobacter invasion antigens) may determine the adherence and invasion abilities of H. cinaedi [7, 22]. Investigating the presence of pathogenic genes in animal H. cinaedi strains, and clarifying their functions would lead to a better understanding pathogenesis of H. cinaedi in both animals and humans.

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