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, septicemia 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*.

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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 bacteraemia. The T22 and T34 strains were isolated from the feces of a healthy dog and hamster, respectively. The *H. cinaedi* CCUG 18818^T strain was obtained from the Culture Collection at the University of Göteborg, Sweden and was used as a positive control for *H. cinaedi*-specific PCR. The *Campylobacter jejuni* 81–176 strain, which 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, 3×10^5 Caco-2 cells were added to each well of a 24-well microplate (ASAHI GLASS Co., Ltd., Tokyo, Japan), and the cells were incubated at 37°C for 24 hr under 5% CO₂ in a humidified atmosphere. An aliquot of 500 µl of bacterial suspension was added to the cells at a multiplicity of infection (MOI) of 100, and the suspension was co-incubated with semiconfluent monolayers of Caco-2 cells with EMEM, which was supplemented with 1% FBS but no antibiotics. The infected monolayers were not centrifuged after inoculation of the bacterial cells. The microplates were incubated at 37°C for 2 hr in a 5% CO₂ humidified atmosphere to allow the bacteria to adhere to and invade the cells. Then, the monolayers were washed three times with 1 ml of EMEM that contained 1% FBS. Fresh EMEM that contained 10% FBS and 250 µg/ml of gentamicin (Sigma-Aldrich Japan) was added to half of the wells for the enumeration of intracellular bacteria after incubation at 37°C for 3 hr in a 5% CO₂ humidified atmosphere. In preliminary experiments, all of the bacterial strains were killed after a 3-hr exposure of 250 µg/ml of gentamicin. Medium without antibiotics was added to the other

half of the wells to enumerate the number of intracellular and extracellular bacteria. Following incubation, the monolayers were washed three times with 100 mM phosphate buffer saline (PBS), pH 7.4 and lysed with 0.01% sodium desoxycholate (Nacalai Tesque). The suspensions were diluted, and viable bacteria were determined by counting the number of colonies on Brucella agar plates that contained 5% defibrinated horse blood after incubation at 37°C for 2–3 days under microaerobic conditions as described above. The number of adhered cells was expressed as the number of intracellular and extracellular bacteria after subtracting the number of intracellular bacteria. Results were expressed as the mean $\pm$ the standard deviation of bacteria adhering to and invading Caco-2 cells for three determinations.

Analysis of epithelial barrier integrity: Approximately 1×10^5 of Caco-2 cells were suspended in EMEM that contained 10% FBS and 50 µg/ml of kanamycin and were seeded onto 0.33-cm² cell culture inserts with a pore size of 3.0 µm (Merck Millipore, Darmstadt, Germany) in 24-well microplates at 37°C for 7–10 days in a 5% CO₂ humidified atmosphere. The medium was changed twice a week. The integrity of tight junctions was monitored by measuring transepithelial electrical resistance (TER) using a Millicell Electrical Resistance System (Merck Millipore). TER was measured as ohms (Ω) $\times$ cm², and fluid resistance was subtracted. When the TER value reached maximal polarity ($>330 \Omega/\text{cm}^2$) after 7–10 days of incubation, the monolayers were washed three times with PBS. Then, approximately $2\text{--}5 \times 10^6$ 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 bacterium 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 $10,000 \times g$ 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 DNeasy 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 distending 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'- CTCGTCCGGATATGGTG -3') and CDT_R (5'- AGAGTTCCTATCACTGC -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 μ l. Each reaction contained 20 pM of each primer (forward and reverse), 200 μ M of each deoxynucleoside triphosphate, 0.5 U of *Taq* DNA polymerase (Qiagen), 1 $\times$ PCR buffer and 2 μ l 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 (acceptation number; 2007-025-6).

Fluorescence microscopy: To observe internalized *H. cinaedi* in Caco-2 cells after invasion, an immunofluorescence procedure was performed. Trypsinized 1×10^5 Caco-2 cells were placed into each well of a chamber slide (Nalgene Nunc, Rochester, NY, U.S.A.) and incubated at 37°C for 3 days under 5% CO₂ in humidified atmosphere. The optical density at 550 nm of the *H. cinaedi* bacterial suspensions (N73, T22 and T34) was adjusted to 0.1 (contained approximately 1×10^7 cfu/ml) in antibiotic-free EMEM, which was supplemented with 10% FBS. Then, 500 μ l of each bacterial suspension was inoculated into the cell monolayers, which were prewashed twice with PBS. The cell monolayers were incubated at 37°C for 24 hr in a 5% CO₂ humidified atmosphere. Then, the cell monolayers were fixed using 4% paraformaldehyde in PBS and permeabilized using 0.1% Triton X-100 (Nacalai Tesque) with 0.5% bovine serum albumin (Sigma-Aldrich Japan). *H. cinaedi* that adhered to and invaded Caco-2 cells were labeled with rabbit anti-*H. cinaedi* serum (1:200) at 25°C for 1 hr, and goat anti-rabbit antibodies conjugated with fluorescein isothiocyanate (FITC) (7.5 μ g/ml) (Invitrogen, Carlsbad, CA, U.S.A.) were added. Rhodamine phalloidin (1 U/ml) (Invitrogen) was added to detect the actin of Caco-2 cells. Internalized bacteria in Caco-2 cells were visualized by three-dimensional analysis using the fluorescence microscope BIOREVO BZ-9000 (KEYENCE, Osaka, Japan).

Statistical analysis: The results were expressed as the mean $\pm$ the standard deviation for every experiment, which were performed in triplicate. The statistical analysis was performed using Student's *t*-test. Statistical significance was defined as $P < 0.05$.

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 (10^6 cfu for adhesion and 10^4 cfu for invasion, respectively) compared with the canine strain T22 (10^5 cfu for adhesion and 10^3 cfu for invasion) and the hamster strain T34 (10^4 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).

Internalization of *H. cinaedi* in Caco-2 cells: To confirm the internalization of *H. cinaedi* in Caco-2 cells, the monolayers were infected with the three *H. cinaedi* strains for 24 hr, and cell internalization was visualized using a fluorescence microscope. The presence of *H. cinaedi* strains in Caco-2 cells was observed in the cross-section of the X-, Y- and Z-axes (Fig. 2). Regardless of the isolate origin, all of the *H. cinaedi* strains exhibited invasion potential in Caco-2 cells.

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

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	1.35×10^8	$^{a)}(1.32 \pm 0.29) \times 10^6$	$(4.40 \pm 0.14) \times 10^4$
T22	dog	4.90×10^7	$(3.38 \pm 1.37) \times 10^5$	$(9.00 \pm 1.44) \times 10^3$
T34	cat	5.27×10^7	$(2.33 \pm 0.81) \times 10^4$	$(1.67 \pm 1.15) \times 10$
81-176	human	6.45×10^7	$(1.59 \pm 0.40) \times 10^6$	$(3.88 \pm 0.42) \times 10^5$

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

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 ($n=3$) $\pm$ 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 a *H. cinaedi*-specific PCR with DNA that was extracted from the basolateral medium. The CCUG 18818^T strain was used as a positive control for *H. cinaedi*-specific PCR.

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 μ m. (A) The N73 strain of human origin in Caco-2 cells, (B) The T23 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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