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# Inability of an Isogenic Urease-Negative Mutant Strain of *Helicobacter mustelae* To Colonize the Ferret Stomach

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**Eight ferrets specific-pathogen-free for *Helicobacter mustelae* were given, per dose, approximately $3.0 \times 10^7$ CFU of either the wild-type parent strain of *H. mustelae* (NCTC 12032) (two ferrets), the isogenic urease-negative mutant strain of *H. mustelae* (10::Tn*3*Km) (four ferrets), or sterile culture broth (two ferrets). Infection status was monitored by endoscopic gastric biopsy for urease activity, histopathology, and culture and by serology at 3, 6, 10, and 21 weeks. All ferrets were necropsied at 25 weeks. Both negative control ferrets remained uninfected, both ferrets receiving the *H. mustelae* wild-type parent strain became infected after two doses of the organism, and all four ferrets given two doses of the isogenic urease-negative mutant strain of *H. mustelae* remained uninfected throughout the 6-month study. Histopathology correlated with infection status. *H. mustelae*-infected ferrets exhibited diffuse mononuclear inflammation in the subglandular region and the lamina propria of the gastric mucosa, while uninfected ferrets showed no or minimal inflammation. These results suggest that urease activity is essential for colonization of the ferret stomach by *H. mustelae*.**

*Helicobacter pylori* infection is known to be a cause of chronic gastritis in humans (1, 2, 47) and is a major risk factor for the development of peptic ulcer disease (29, 44, 46) and gastric adenocarcinoma (4, 18, 40, 43). Several determinants of pathogenicity in *H. pylori* infection, including flagellar motility (35, 49), vacuolating cytotoxin (VacA) (6, 17, 38, 39, 45), various adhesins (9, 14), the cytotoxin-associated gene A (CagA) antigen (5, 7, 8, 52, 53), and bacterial urease activity (15, 31, 32, 33), have been proposed. Although a number of virulence factors have been implicated in the establishment of infection and the development of gastritis and ulcers (34, 37, 54), limited experimental evidence is available to prove or disprove their association with observed gastrointestinal pathology.

Initial studies conducted in the gnotobiotic piglet suggested that virulence correlated with motility but not as well with cytotoxin production (13) and that urease activity (11) was essential for gastric colonization. While providing enticing results, these studies were limited in that the bacterial strains used to infect the piglets were not genetically characterized and the studies allowed only an analysis of acute colonization and infection because of the time limitations (approximately 30 days) of maintaining piglets in the gnotobiotic state (36). Recently, isogenic urease-negative mutant strains of *H. pylori* have been constructed and administered to gnotobiotic piglets (12) and nude mice (51). Both of these studies indicated that urease activity was essential for colonization of the stomach. However, these species are not the natural hosts for *H. pylori*, and the studies were of short duration (1 to 4 weeks). Additional limitations of these models include the immunologically compromised state of the nude mouse and the germ-free state of the piglets, which does not mimic the natural gastric environment of a host colonized with a variety of gastrointestinal flora.

An animal model that does not suffer from the limitations of the gnotobiotic piglet or the nude mouse models and has many similarities with *Helicobacter* disease in humans is the *H. mustelae* ferret model developed in our laboratory (19–21, 23–25, 27). Ferrets are naturally colonized with *H. mustelae* soon after birth and remain infected throughout their life, allowing longitudinal investigation of all stages of infection and the progression of disease. Koch's postulates have been fulfilled, establishing *H. mustelae* as a gastric pathogen for ferrets (26). *H. mustelae* infection universally causes gastritis in the ferret and is associated with gastric and duodenal ulcers (25, 30, 50). *H. pylori* and *H. mustelae* are the only gastric organisms known to adhere to the surface of gastric mucosa and to be associated with peptic ulceration (3, 41). The ferret model has also been used to investigate therapeutic protocols for the eradication of *Helicobacter* infection (42) and to study the cocarcinogenicity of *Helicobacter* infection in the development of gastric adenocarcinoma in ferrets administered *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (28).

Several of the virulence factors of *H. pylori* have also been identified in *H. mustelae* (10, 16, 49). Recently, an isogenic urease-negative mutant strain of *H. mustelae* has been constructed (48). In this report, we describe experiments designed to investigate whether an isogenic urease-negative mutant strain of *H. mustelae* colonizes the gastric mucosa of the ferret (*Mustela putorius furo*) following oral inoculation.

Two strains of *H. mustelae* were used in this study. The wild-type strain, *H. mustelae* NCTC 12032 (National Collection of Type Cultures, London, England), was administered to the positive control ferrets and also served as the parent strain for construction of the isogenic urease-negative mutant strain, *H. mustelae* 10::Tn*3*Km (48). Shuttle mutagenesis and allelic exchange were performed on the wild-type strain to generate a mutant strain which carried a 1.8-kb mini-Tn*3*-Km transposon insertion in the *ureB* gene. Phenotypic characterization of the isogenic urease-negative mutant strain included analysis of the urease activity and the pH sensitivity of the strain. In vitro growth characteristics were identical to those of the parent strain. Immunoblotting for the urease protein subunits indicated that the mutant strain produced no detectable urease and expressed no UreB and reduced amounts of UreA.

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Both *H. mustelae* strains were grown for 72 h on Trypticase soy agar containing 5% sheep blood (TSAII–5% SB; BBL Microbiology Systems, Cockeysville, Md.) at 37°C in vented jars containing 90% $N_2$, 5% $H_2$, and 5% $CO_2$. All cultures were catalase and oxidase (Oxidase Reagent Droppers; BBL Microbiology Systems) positive. Gram staining and phase-contrast microscopy indicated morphology consistent with *H. mustelae*. The parent strain was urease positive (Urea Agar Slant; BBL Microbiology Systems), while the isogenic mutant strain was urease negative. Kanamycin resistance was assayed by plating on TSAII–5% SB plates with 30 μg kanamycin discs (Sensi-Disc; BBL Microbiology Systems). The parent strain was sensitive to kanamycin, while the isogenic urease-negative mutant strain was resistant to kanamycin. Organisms were harvested from plates into brucella broth (Difco Laboratories, Detroit, Mich.) with 30% glycerol and kept on ice prior to administration to ferrets. A sample was taken to determine approximate concentrations of organisms by optical density. Biopsy samples were homogenized in 1.0 ml of phosphate-buffered saline and plated on TSAII–5% SB plates and brucella blood agar-TVP (trimethoprim-vancomycin-polymyxin B) plates (Remel, Lenexa, Kans.). Cultures were checked every 3 days for 14 days for growth (26) and kanamycin resistance.

Eight ferrets specific-pathogen-free for *H. mustelae* were chosen from the specific-pathogen-free ferret colony maintained by the Division of Comparative Medicine at the Massachusetts Institute of Technology. The ferrets were all 13 months old at the start of the study. Group I (negative control) consisted of one male and one female ferret which received sterile broth. Group II (positive control) also consisted of one male and one female ferret which received the *H. mustelae* wild-type parent strain. Group III (test group) consisted of two male and two female ferrets which received the isogenic urease-negative mutant strain. Ferrets were housed individually in stainless steel (Research Equipment Co., Bryan, Tex.) or plastic (MediCage; Lock Solutions, Inc., Kenilworth, N.J.) cages. Groups of ferrets were housed in separate cubicles or rooms to prevent cross-contamination between groups. Ferrets were given water and ferret chow (Lab Diet 5L14; PMI Feeds, Inc., St. Louis, Mo.) ad libitum.

Baseline endoscopic gastric biopsies to confirm the specific-pathogen-free status of the ferrets were performed as described previously (42). Two milliliters of blood was collected by jugular venipuncture for *H. mustelae* immunoglobulin G antibody titers (21, 22, 25). Three 2-mm pinch biopsies of the gastric mucosa at the pyloric antrum and the gastric body were obtained for culture, histology, and urease plate assay (32). Gastric tissues were processed routinely in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histopathology and with Warthin-Starry silver stain to demonstrate *H. mustelae* organisms.

Three milliliters of broth containing approximately $10^7$ CFU of the appropriate strain of *H. mustelae* organisms per ml was given to each anesthetized ferret in groups II and III as described previously (26). Ferrets in group I received 3 ml of sterile broth. Dosing was performed on weeks 1 and 17. Ferrets were biopsied and sera were collected on weeks 3, 6, 10, and 21/22. All ferrets were euthanatized on week 25. At necropsy, stomachs were mapped by culture and urease plate assay by sampling nine different regions of the gastric mucosa, three sites each from the cardia, body, and antrum.

Prior to inoculation of the *H. mustelae* strains, all eight ferrets were confirmed to be uninfected with *H. mustelae* on the basis of urease assay, serology, culture, and histopathology (Table 1). One ferret in group II (positive control) had a positive urease response; however, bile reflux tinged with blood was noted in this ferret during endoscopy. Ferrets in group I received sterile broth and remained uninfected throughout the study (Table 1). Neither ferret had any positive responses by any of the four methods used to detect the presence of *H. mustelae* organisms. Ferrets in group II received two doses (approximately $3.0 \times 10^7$ CFU per dose) of the wild-type parent strain, *H. mustelae* NCTC 12032, and became infected with the organism (Table 1). One ferret became infected following the first oral dosing, while the other ferret became infected following the second oral dosing. Ferrets in group III received two doses (approximately $3.0 \times 10^7$ CFU per dose) of the isogenic urease-negative mutant strain, *H. mustelae* 10::Tn*3*Km. All four ferrets in this group remained uninfected, as determined by culture and histopathology, throughout the study (Table 1). Two ferrets had low serologic responses (1:84 and 1:90) at the 10-week biopsy, and one ferret had a positive urease response at the 3- and 6-week biopsies.

Histopathology in ferrets in groups I and III was limited to minimal numbers of lymphocytes and an occasional neutrophil scattered throughout the subglandular portion of cardiac, body, and antral mucosa. Warthin-Starry staining revealed no evidence of *Helicobacter* organisms. Histopathology in ferrets

TABLE 1. Results from isogenic urease-negative mutant *H. mustelae* infection study

| Time and Procedure | No. positive[a] | | |
|---|---|---|---|
| | Group I (negative control; $n = 2$) | Group II (positive control; $n = 2$) | Group III (test group; $n = 4$) |
| Baseline biopsy | | | |
| Culture | 0 | 0 | 0 |
| Urease | 0 | 1 | 0 |
| Serology | 0 | 0 | 0 |
| Histopathology | 0 | 0 | 0 |
| Week 1, 1st dose[b] | | | |
| Week 3, 1st biopsy | | | |
| Culture | 0 | 1 | 0 |
| Urease | 0 | 1 | 1 |
| Serology | 0 | 0 | 0 |
| Histopathology | 0 | 1 | 0 |
| Week 6, 2nd biopsy | | | |
| Culture | 0 | 1 | 0 |
| Urease | 0 | 2 | 1 |
| Serology | 0 | 1 | 0 |
| Histopathology | 0 | 1 | 0 |
| Week 10, 3rd biopsy | | | |
| Culture | 0 | 1 | 0 |
| Urease | 0 | 1 | 0 |
| Serology | 0 | 1 | 2 |
| Histopathology | 0 | 1 | 0 |
| Week 17, 2nd dose[b] | | | |
| Week 21/22, 4th biopsy | | | |
| Culture | 0 | 2 | 0 |
| Urease | 0 | 2 | 0 |
| Serology | 0 | 2 | 0 |
| Histopathology | 0 | 2 | 0 |
| Week 25, necropsy | | | |
| Culture | 0 | 2 | 0 |
| Urease | 0 | 1 | 0 |
| Serology | 0 | 2 | 0 |
| Histopathology | 0 | 2 | 0 |

[a] Positive results were defined as follows: growth of organisms within 2 weeks (culture), color change within 24 h (urease), titer greater than 1:64 (serology), and organisms visible on Warthin-Starry-stained sections (histopathology).

[b] Animals were administered broth (group I), *H. mustelae* NCTC 12032 (group II), or *H. mustelae* 10::Tn*3*Km (group III).

in group II consisted of a mild diffuse leukocytic infiltrate in the subglandular portion of the cardiac and antral mucosa composed primarily of lymphocytes and occasional neutrophils. There were also focal lymphoid aggregates and small clusters of leukocytes scattered throughout the cardiac and antral lamina propria. Minimal lymphocytic infiltration was present in the subglandular portion of the body mucosa. Presence of *H. mustelae* organisms coincided with the severity of inflammation. Moderate numbers of organisms were observed within the crypts of glands and the gastric pits throughout the antral mucosa and scattered throughout the cardiac mucosa, with fewer organisms being present in the body mucosa.

To investigate the role of urease in the colonization of the gastric mucosa by *Helicobacter* organisms, we used an isogenic urease-negative mutant strain of *H. mustelae* to infect the organisms' natural host, the ferret. The isogenic urease-negative mutant strain of *H. mustelae* was unable to colonize, while the wild-type parent strain was able to infect the ferret stomach (Table 1). Our criteria for infection were culture, histopathology, serology, and urease assay, in descending order of sensitivity and specificity.

The urease assay (32) was performed to detect infection by the parent strain in group II and contamination by a wild-type strain in groups I and III. In our experience, the urease plate assay is not very specific, as intestinal reflux and blood from biopsy samples may give false-positive results. The urease plate assay also has low sensitivity and requires a significant number of organisms to show a response. This may be the explanation for the negative urease results at 25 weeks in one ferret in group II that had been infected following the first administration of the organisms and had organisms present on histopathology.

The serologic assay that we have developed for *H. mustelae* infection uses whole-cell sonic extracts as the antigen. Ferrets usually develop an immunoglobulin G antibody response 4 to 6 weeks following infection (26). In this study, one ferret in group II had a consistently high titer (1:675 to 1:1,024) beginning at 6 weeks and lasting through 25 weeks. The other ferret in group II developed a moderate immunoglobulin G response (1:416) at 21 weeks, 4 weeks after administration of the second dose of organism. This ferret's titer declined to 1:84 at 25 weeks, although this was still considered positive with a cutoff of 1:64. Two ferrets in group III had low titers (1:84 and 1:90) at the 10-week biopsy but were negative at all other time points. Since viable *H. mustelae* organisms of the isogenic mutant strain were orally inoculated into the group III ferrets, it is possible that these organisms elicited an immune response to *H. mustelae* antigens even though the ferrets did not become colonized.

The inability of urease-negative *Helicobacter* organisms to colonize the gastric mucosa of several species confirms the important role of urease activity in colonization; however, the mechanism by which urease allows colonization has yet to be determined. Urease activity was thought to neutralize the gastric acid immediately surrounding the organism until it could enter the gastric mucus (31). A recent study in achlorhydric gnotobiotic piglets discounted this hypothesis because urease-negative *H. pylori* organisms were found only in small numbers ($10^2$ CFU) and considered unable to colonize (12). This study also discounted the theory that urease may play a nutritional role by promoting nitrogen metabolism (31) by finding that only the wild-type strain was able to colonize following administration of both wild-type and mutant strains in a cocolonization experiment (12).

In summary, although the mechanism by which urease allows the colonization of the gastric mucosa by *Helicobacter* organisms remains unknown, we have shown that in its natural host, an isogenic urease-negative mutant strain of *H. mustelae* was unable to colonize the ferret stomach.

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