

In Situ Molecular Diagnosis and Histopathological Characterization of Enteroadherent *Enterococcus hirae* Infection in Pre-Weaning-Age Kittens[∇]

Jodi L. Nicklas,¹ Peter Moisan,² Maria R. Stone,¹ and Jody L. Gookin^{1*}

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606,¹ and Rollins Animal Disease Diagnostic Laboratory, Raleigh, North Carolina 27699²

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The bacterial causes of diarrhea can be frustrating to identify, and it is likely that many remain undiagnosed. The pathogenic potential of certain bacteria becomes less ambiguous when they are observed to intimately associate with intestinal epithelial cells. In the present study we sought to retrospectively characterize the clinical, *in situ* molecular, and histopathological features of enteroadherent bacteria in seven unrelated kittens that were presumptively diagnosed with enteropathogenic *Escherichia coli* (EPEC) on the basis of postmortem light microscopic and, in some cases, microbiological examination. Characterization of the enteroadherent bacteria in each case was performed by Gram staining, *in situ* hybridization using fluorescence-labeled oligonucleotide probes, PCR amplification of species-specific gene sequences, and ultrastructural imaging applied to formalin-fixed paraffin-embedded sections of intestinal tissue. In only two kittens was EPEC infection confirmed. In the remaining five kittens, enteroadherent bacteria were identified as *Enterococcus* spp. The enterococci were further identified as *Enterococcus hirae* on the basis of PCR amplification of DNA extracted from the formalin-fixed, paraffin-embedded tissue and amplified by using species-specific primers. Transmission electron microscopy of representative lesions from *E. coli*- and *Enterococcus* spp.-infected kittens revealed coccobacilli adherent to intestinal epithelial cells without effacement of microvilli or cup-and-pedestal formation. Enterococci were not observed, nor were DNA sequences amplified from intestinal tissue obtained from age-matched kittens euthanized for reasons unrelated to intestinal disease. These studies suggest that *E. hirae* may be a common cause of enteroadherent bacterial infection in pre-weaning-age kittens and should be considered in the differential diagnosis of bacterial disease in this population.

Diarrhea is a frequent clinical sign at the time of death or euthanasia of kittens, particularly those residing in shelters or rescue facilities. Enteritis is second only to the specific diagnosis of feline parvovirus infection as the most common cause of kitten mortality identified by histopathology-based studies (2). Aside from viral, protozoal, and helminthic causes of enteritis, the bacterial culprits of diarrhea are particularly problematic to identify. The intestinal tract harbors a diverse population of bacteria that play critical roles in nutrient assimilation, mucosal immunity, and colonization resistance. Recognition of pathogenic bacteria within this population is hampered by our limited knowledge of normal bacterial diversity, the challenge of distinguishing commensal from pathogenic bacteria, the frequent presence of pathogenic bacteria in clinically normal animals, and the ability of commensal bacteria to become pathogenic in genetically susceptible individuals or under abnormal environmental conditions. The pathogenic potential of certain bacteria becomes less ambiguous when they are observed to intimately adhere to intestinal epithelial cells.

There are few reports of enteroadherent bacterial infection in cats. Most reported cases involve the cultivation from feces of *Escherichia coli* that are molecularly characterized as con-

taining the gene coding for intimin (*eae*) (10, 11, 16). Bacterial expression of intimin and the translocated intimin receptor (*tir*) mediates adherence of enteropathogenic and some enterohemorrhagic strains of *E. coli* to the intestinal epithelium. This attachment is accompanied by effacement of the epithelial microvilli and the rearrangement of actin to form membranous projections beneath the bacteria resembling a cup and pedestal. Attaching and effacing lesions have been described in only two cats; however, images of the lesions were not provided (18).

As a prelude to prospective studies examining the clinical importance of enteropathogenic *E. coli* (EPEC) infection in kittens, we sought to retrospectively characterize the clinical, *in situ* molecular, and histopathological features of enteroadherent bacteria in seven unrelated kittens that were presumptively diagnosed with EPEC on the basis of light microscopic and, in some cases, microbiological examination. Our unexpected discovery that most of these kittens were infected with enteroadherent *Enterococcus hirae* and not *E. coli* has important implications for future selection and interpretation of microbiological tests in these cases.

MATERIALS AND METHODS

Intestinal samples. Formalin-fixed, paraffin-embedded intestinal tissue from seven unrelated kittens submitted to a state animal disease diagnostic laboratory for necropsy examination from 2004 to 2008 were retrospectively obtained. In each case, light microscopic examination of the small intestine revealed mild-to-moderate, subacute-to-acute, necrotizing enterocolitis with extensive colonization of the small intestinal epithelium by adherent coccobacilli. On the basis of

* Corresponding author. Mailing address: North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, NC 27606. Phone: (919) 513-6295. Fax: (919) 513-6538. E-mail: Jody_Gookin@ncsu.edu.

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these findings a diagnosis of attaching and effacing *E. coli* infection was presumed. The kittens ranged in age from 3 to 10 weeks (median age, 6 weeks) and were each being housed in shelter or foster facilities. Clinical signs prior to death or euthanasia included diarrhea and lethargy (four kittens), upper respiratory tract infection (one kitten), sudden death (one kitten), and unknown (one kitten). Aerobic bacterial culture of feces was performed at the time of necropsy for four kittens, three of which were positive for *E. coli*. Genomic DNA extracted from selected *E. coli* colonies, was assayed by multiplex PCR for the presence of genes encoding intimin (*eae*), *Shiga* toxins (*Stx1* and *Stx2*), heat-stable toxins (*STa* and *STb*), heat-labile toxin (*LT*), or cytotoxic necrotizing factors (*CNF-1* and *CNF-2*) as previously described (9, 23). Two kittens were identified with *eae*-positive nonhemolytic *E. coli*, one kitten with *CNF-1* and *CNF-2*-positive beta-hemolytic *E. coli*, and one kitten was culture-negative for *E. coli*. Based on the presumption that enterococci were normal flora, neither their presence nor their identity was reported.

For comparative purposes, formalin-fixed, paraffin-embedded intestinal tissues were prospectively obtained from an equal number of age-matched control kittens, euthanized by a local animal control facility for reasons unrelated to intestinal tract disease.

FISH. Formalin-fixed, paraffin-embedded tissue samples were sectioned at a thickness of 4 μ m and mounted on poly-L-lysine-coated slides. The tissue sections were deparaffinized, rehydrated, and air dried prior to hybridization with fluorescence *in situ* hybridization (FISH) probes at a working strength of 5 ng/ μ l (14). The universal bacterial probe Eub338 (14), labeled at the 3' end with 6-FAM, was used to identify eubacteria. For subsequent analyses, specific probes directed against *E. coli/Shigella* (14) or *Enterococcus* spp. (Enc221, 5'-Cy3-CAC CGCGGGTCCATCATCA-3') were simultaneously applied. The genus-specific *Enterococcus* sp. probe Enc221 has been demonstrated to have an analytical specificity of 100% when tested against 14 different *Enterococcus* species and 19 nonenterococcal reference strains (25). Probe specificity was additionally evaluated by using a sense probe non-Enc221 (5'-6FAM-TGATGGATGGACCCGC GGTG-3') and by including positive and negative control slides in each assay. Positive control slides included formalin-fixed, paraffin-embedded intestinal tissue from a pig and dog with culture and multiplex PCR-confirmed *eae*-positive, enteropathogenic *E. coli* infection and a pig with intestinal *Enterococcus durans* infection. For the hybridization of Eub338 and Enc221 probes to enterococci, optimal permeabilization was achieved by washing the slides in permeabilization buffer (100 mM Tris-HCl [pH 7.5], 50 mM EDTA) followed by treatment with lysozyme (L6876 [Sigma Chemical Company, St. Louis, MO]; 10 mg/ml in permeabilization buffer) for 30 min at 37°C in a humidified chamber. Slides were subsequently washed in diethylpyrocarbonate-treated deionized water, air dried, and hybridized as previously described (14).

DNA extraction from formalin-fixed, paraffin-embedded intestinal tissue. Intestinal tissue (~80 mg), corresponding in location to the site of microscopically observed enteroadherent bacteria, was excised from the paraffin block of each infected and all control kittens. Between each tissue block, an equivalent amount of paraffin was excised from a block devoid of tissue to assess for the possibility of cross-contamination between samples. The excised tissue samples were added to 200 μ l of ATL buffer (Qiagen, Valencia, CA) and microwaved at 5-s intervals until the paraffin liquefied. The solution was centrifuged (150 \times g for 10 min), followed by removal of the paraffin ring by using a sterile pipette tip. The remaining solution was vortexed for 5 min (vortex adaptor; MoBio, Carlsbad, CA) in the presence of ZR BashingBeads (Zymo Research, Orange, CA), treated with 100 μ l of lysozyme (40 mg/ml; Sigma), and incubated for 7 h at 37°C. The solution was subsequently treated with proteinase K (20 μ l) and incubated overnight at 56°C, followed by the addition of 200 μ l of AL buffer (Qiagen) and incubation at 55°C for 30 min and at 95°C for 15 min. The remaining protocol, beginning with the addition of 200 μ l of ethanol, was performed as described for the DNeasy blood and tissue kit (Qiagen). Extractions performed concurrently with the feline samples included paraffin wax spiked with *in vitro*-cultured *E. hirae* ATCC 8043 (positive control) and extraction reagents alone (negative control).

DNA extraction from *E. hirae* and *E. durans* culture isolates. *E. hirae* (ATCC 8043) and *E. durans* (ATCC 6056) type strains were purchased from the American Type Culture Collection and then reconstituted and cultured for 24 h at 37°C in brain heart infusion medium. Bacteria were pelleted from 1 ml of broth culture and pretreated with 180 μ l of lysozyme (20 mg/ml in PCR-grade water) at 37°C for 30 min. DNA was extracted by using a commercially available kit (DNeasy blood and tissue kit; Qiagen) in accordance with recommendations for Gram-positive bacteria.

Identification of *E. hirae* and *E. durans* by PCR amplification of species-specific muramidase gene sequences. All samples of DNA extracted from paraffin-embedded intestinal tissue, as well as paraffin blocks devoid of tissue (negative controls), were first subjected to PCR amplification of an ~400-bp gene

sequence of feline GAPDH (glyceraldehyde-3-phosphate dehydrogenase) DNA as previously described (12). Subsequent PCR was performed using the species-specific primer pairs *mur-2_{ed}* (*E. durans*) and *mur-2* (*E. hirae*), which were synthesized (IDT Technologies, Coralville, IA) in accordance with published sequence data (1). Reaction conditions for *mur-2_{ed}* and *mur-2* gene amplification were as follows: a 100- μ l reaction volume of PCR buffer II (Perkin-Elmer, Foster City, CA) containing 2 U of AmpliTaq Gold DNA polymerase, 2 mM MgCl₂, 70 pmol of each primer, 200 μ M each deoxynucleotide triphosphate, and 10 μ l of DNA template. DNA amplification was performed in a Bio-Rad iCycler at the following temperature profiles: initial denaturation at 95°C for 5 min; denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min for 50 cycles; followed by a final extension for 10 min at 72°C. Amplicons were visualized by UV illumination after electrophoresis of 10 μ l of the reaction solution in a 1.5% agarose gel containing ethidium bromide. Reaction products were purified (QIAquick PCR purification kit; Qiagen) and sequenced by a commercial laboratory (Davis Sequencing, Davis, CA).

Transmission electron microscopy. Intestinal tissue (~80 mg), corresponding in location to the site of microscopically observed enteroadherent bacteria, was cut from the original paraffin block of three kittens. Excess paraffin was trimmed away, and the remaining tissue deparaffinized overnight in xylene. The tissue samples were rehydrated in a graded series of alcohol (100%, twice for 30 min each time; 95%, twice for 15 min each time; 75%, once for 15 min; 50%, once for 15 min), transferred to 0.1 M phosphate buffer (pH 7.2 to 7.4), and fixed in 1% osmium tetroxide for 1 h. Tissue samples were then dehydrated in a graded series of alcohol (50%, once for 15 min; 75%, once for 15 min; 95%, twice for 15 min each time; 100%, twice for 30 min each time), fixed in acetone (2 \times 10-min) and embedded in Spurr resin. Ultrathin sections were placed on 200-mesh copper grids, post-stained with uranyl acetate and lead citrate, and examined with a FEI/Philips EM 208S transmission electron microscope.

RESULTS

Light microscopic findings in kittens with enteroadherent bacterial infection and in control kittens. In the small intestines of all seven kittens extensive colonies of coccobacilli were present in diplococcal and palisading formations over the luminal surface (Fig. 1). In one kitten the colon was also involved. The organisms were tightly adherent to enterocytes of the villous tips, as well as to necrotic enterocytes within the luminal detritus. Affected cells were shrunken, hyper eosinophilic, and often contained pyknotic nuclei. Within the superficial and deep lamina propria only occasional neutrophils and small numbers of lymphocytes were observed. In three kittens concurrent intestinal pathogens were identified, including *Tritrichomonas foetus*, panleukopenia virus, and adenovirus (1 kitten each). Enteroadherent bacteria were not observed in intestinal tissue samples obtained from seven age-matched control kittens euthanized for reasons unrelated to gastrointestinal disease.

FISH using eubacterial and *E. coli/Shigella*-specific oligonucleotide probes. Because each kitten had been presumptively diagnosed with enteroadherent *E. coli* infection on the basis of light microscopy, *in situ* hybridization was initially performed using individual and simultaneously applied eubacterial (Eub338) and *E. coli/Shigella*-specific oligonucleotide probes. Hybridization of both probes to the enteroadherent bacteria was observed for only two kittens (Fig. 2). Identical results were obtained with intestinal tissue from a dog and pig diagnosed with *eae*-positive *E. coli* infection. For the remaining five kittens, neither probe hybridized to the enteroadherent bacteria. *E. coli* was also not observed by FISH in small intestinal tissues from age-matched control kittens, with the exception of one case in which a few *E. coli* organisms were observed in the intestinal lumen.

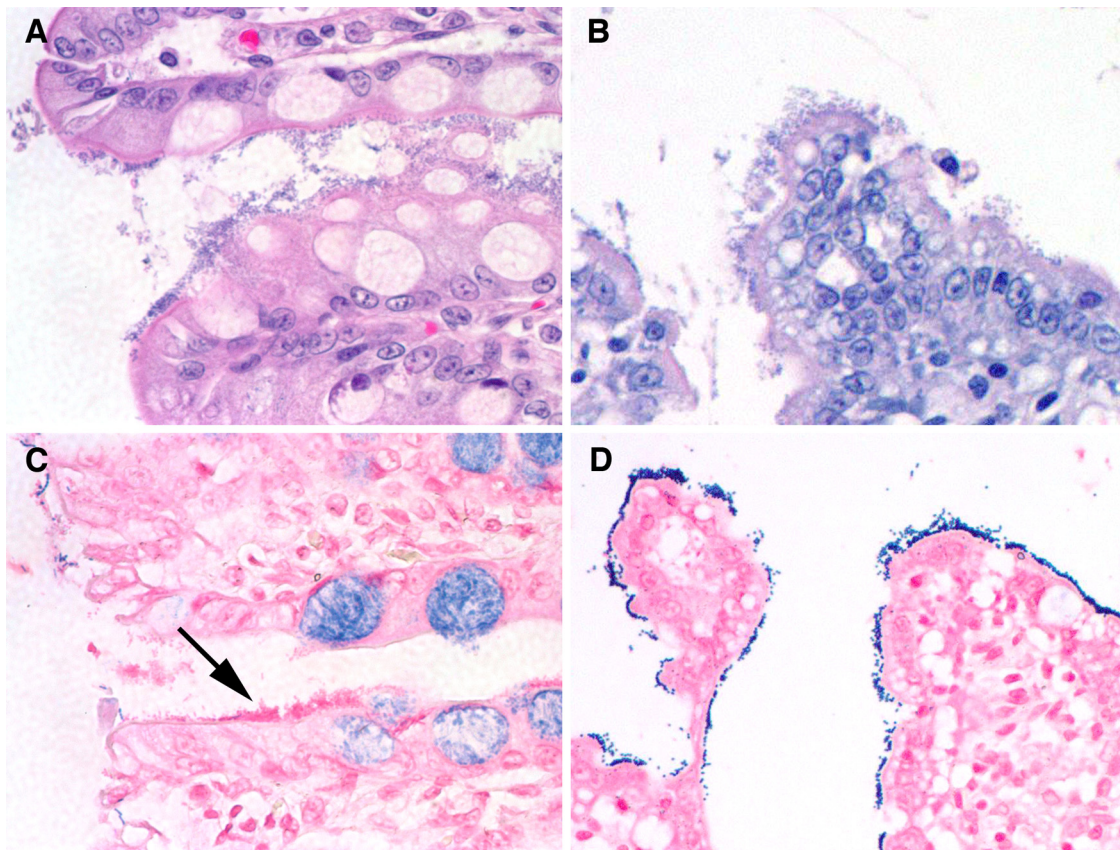


FIG. 1. Light microscopic appearance of the small intestinal epithelium from a kitten with enteroadherent *E. coli* (A) and enteroadherent *Enterococcus* (B) infection after staining with hematoxylin and eosin. Note the similarity in the histological appearance of the bacteria. After Gram staining of the tissue sections, bacteria from a cat with *E. coli* stain negative (C; arrow), whereas those from the cat with *Enterococcus* stain positive (D).

Characterization of enteroadherent bacteria by tissue Gram staining. To determine whether failure of probes to hybridize to enteroadherent bacteria in the remaining five kittens could be attributed to the presence of Gram-positive organisms, intestinal tissue from all kittens were subjected to Gram staining. Enteroadherent bacteria in the two kittens for which positive hybridization with eubacterial and *E. coli/Shigella* probes were observed stained negative by Gram staining. In the remaining five kittens in which neither probe hybridized, enteroadherent bacteria stained Gram positive (Fig. 1). Only small numbers of Gram-negative rods and rare Gram-positive coccobacilli were observed in the small intestinal lumens of control kittens.

FISH using eubacterial and *Enterococcus*-specific oligonucleotide probes. To determine the genus of Gram-positive bacteria responsible for enteroadherent infection in the remaining five kittens, *in situ* hybridization was performed using individual and simultaneously applied eubacterial (Eub338) and *Enterococcus* sp.-specific oligonucleotide probes after pretreatment of tissue sections with lysozyme. Hybridization of both eubacterial and *Enterococcus* probes were observed in the five kittens with enteroadherent Gram-positive bacterial infection (Fig. 3) and in intestinal tissue from a pig with *E. durans* infection. In the two kittens with enteroadherent Gram-negative *E. coli* infection, hybridization of only the eubacterial probe was observed. Positive hybridization was not observed

when Gram-positive bacteria were probed with a negative control, fluorescence-labeled sense oligonucleotide for the *Enterococcus* probe or the *E. coli/Shigella* probe. Small numbers of enterococci were observed in the small intestinal lumens of two age-matched control kittens.

Identification of enteroadherent bacteria as *E. hirae*. Both *E. hirae* and *E. durans* have been variably cultured from the feces of puppies, calves, foals, piglets, and suckling rats having diarrhea associated with intestinal epithelial colonization by Gram-positive bacteria (3, 4, 6–8, 17, 20, 22, 24). In none of these cases however, were the enteroadherent bacteria definitively identified as enterococci. To unambiguously determine the identity of the enteroadherent organisms in the kittens examined in the present study, infected sections of intestine were selectively excised from each paraffin block for DNA extraction and PCR amplification using *E. durans*- and *E. hirae*-specific primers. Robust amplification of a 400-bp fragment of the feline GAPDH gene from all blocks containing tissue demonstrated that quality DNA was extracted from each sample. Subsequent PCR performed on DNA extracted from intestinal tissues of four of the five kittens with enteroadherent enterococci resulted in amplification of a 521-bp product corresponding in size to the target sequence of the *E. hirae* muramidase gene. Sequence analysis of the PCR amplicons revealed 99% sequence identity to that of *E. hirae* (accession no.

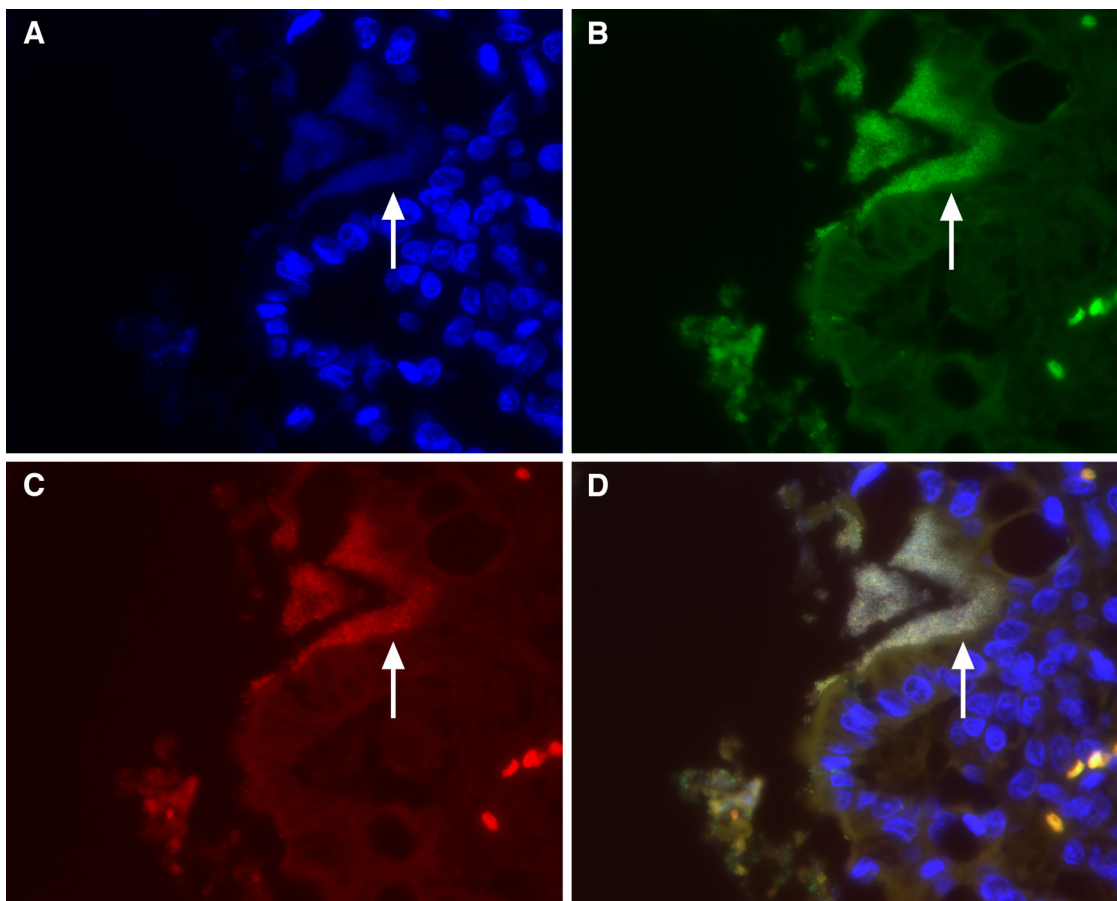


FIG. 2. Fluorescence *in situ* hybridization (FISH) of the small intestinal epithelium from a kitten with enteroadherent *E. coli* infection. (A) The nuclear counterstain, DAPI (4',6'-diamidino-2-phenylindole), enables visualization of intestinal epithelial and lamina propria nuclei. Nucleic acid within the enteroadherent bacteria (arrow) can be seen in the lumen along the junction of two intestinal villi. (B) FISH using the eubacterial probe Eub338 labeled with FAM (green fluorescence) identifies the bacteria as eubacteria. (C) FISH using an *E. coli/Shigella*-specific probe labeled with Cy3 (red fluorescence) identifies the eubacteria as *E. coli/Shigella*. (D) Merged image demonstrating the simultaneous hybridization (light yellow) of enteroadherent bacteria with eubacterial and *E. coli/Shigella*-specific oligonucleotide probes.

M77639). Amplification of products corresponding in size to the target sequence of the *E. durans* muramidase gene homologue (177 bp) was not observed for any samples. Further, neither *E. hirae* or *E. durans* gene sequences were amplified from paraffin blocks serving as extraction controls, the paraffin block of a kitten infected with enteroadherent *E. coli*, or those of the control kittens in which enteroadherent bacteria were not identified.

Transmission electron microscopy. To characterize the ultrastructural relationship of *E. hirae* with the intestinal epithelium and compare these findings to those of enteroadherent *E. coli*, representative lesions from *E. coli*- and *E. hirae*-infected kittens and an *E. coli*-infected pig were excised from paraffin-embedded intestinal tissue, processed, and examined by transmission electron microscopy. The ultrastructural relationship between intestinal epithelial cells and enteroadherent *E. coli* or *E. hirae* were similar (Fig. 4). In both infections bacteria were observed to intimately attach to the brush border of the microvillus tips. In the case of *E. hirae*, this attachment was associated with fine electron-dense filaments, which radiated from the surface of the cell wall to microvilli and adjacent

bacteria. In neither case was classic cup-and-pedestal formation or effacement of the microvillus architecture observed.

DISCUSSION

Enterococci are motile, Gram-positive bacteria that reside in the feces of most healthy humans and animals, where they are considered to be members of the normal intestinal flora. In fact, recent analysis of microbial diversity of the feline intestine using 16S rRNA gene sequence analysis identified *Enterococcus* spp. as one of the predominant bacterial species isolated from the jejunum, colons, and feces of healthy young-adult cats (19). Gram-positive cocci, including *Enterococcus* spp., are rarely implicated as a cause of diarrhea. Therefore, it is not surprising that efforts were not taken to specifically culture or identify enterococci from feces of these kittens at the time of necropsy. Particular members of the enterococci, however, have been sporadically implicated as a cause of diarrhea in suckling animals of several host species, including piglets, calves, foals, rats, puppies, and a kitten (3, 4, 6–8, 17, 20, 22, 24). In these cases, light microscopic examination of the

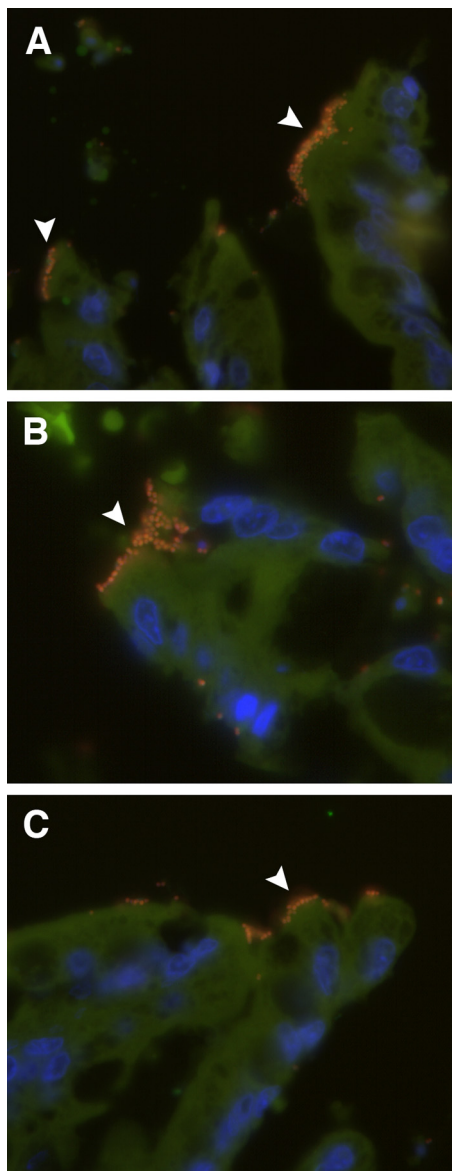


FIG. 3. FISH of small intestinal villous epithelium from kittens with enteroadherent *Enterococcus* infection. Merged images demonstrate the simultaneous hybridization of enteroadherent bacteria with DAPI (blue) eubacterial (green) and *Enterococcus*-specific oligonucleotide probes (red), which generate an orange fluorescence.

small intestine revealed the adhesion of Gram-positive cocci to the apical surface of enterocytes in association with cultivation from feces of *E. durans* or *E. hirae*. However, in no case were the adherent bacteria specifically identified as *Enterococcus* spp.

Identification of the enteroadherent bacteria as enterococci in the kittens described here was unexpected. In each case, *E. coli* infection was presumed on the basis of light microscopic examination of intestinal tissue, and in three cases this was supported by culture from feces of a necrotoxicogenic or attaching and effacing strain of *E. coli*. In fact, the light microscopic appearance of *E. coli* versus *Enterococcus* infection in these kittens was virtually indistinguishable. It is unclear why a Gram

stain was not initially considered in the diagnostic approach to these cases, other than the fact that enteroadherent *Enterococcus* spp. have only rarely been reported in cats (13, 17). Due to our false presumption that the adherent bacteria were *E. coli*, we did not consider an alternate identity for the organisms until they failed to hybridize to the eubacterium-specific oligonucleotide probe Eub338 by FISH. Treatment of tissue sections with the Gram-positive cell wall lytic enzyme, lysozyme, was required for successful hybridization of oligonucleotide probes to the enterococci. By using FISH, our studies unambiguously demonstrated that the adherent bacteria belong to the genus *Enterococcus*. Further, their species was identified as *E. hirae* on the basis of PCR amplification of DNA extracted directly from the site of intestinal lesions. This approach is superior to the historical identification of enteroadherent bacteria on the basis of fecal culture, particularly because *E. hirae* is second only to *E. faecalis* as the most common species of *Enterococcus* isolated from the feces of clinically normal cats (5). Given its high prevalence in normal cats, the use of fecal culture for diagnosis of clinical disease caused by *E. hirae* is likely to be problematic. Likewise, presumptive identification of enteroadherent bacteria on the basis of positive culture results for *E. coli* can also be misleading because a necrotoxicogenic and attaching and effacing strain of *E. coli* was cultured from the feces of two cats with enteroadherent *E. hirae* infection in the present study.

The most characteristic feature of enteroadherent enterococcal infection was an extensive colonization of small intestinal villous epithelial cells by Gram-positive bacteria. The ability to adhere to the intestinal epithelium is reported to be a virulence attribute of only some strains of enterococci, including *E. hirae*, *E. durans*, *E. villorum*, and *E. faecium* (7, 8, 13, 24). Under stressful growth conditions enterococci have been demonstrated to produce binding proteins that mediate adhesion to the intestinal epithelium (21). Ultrastructurally, this adhesion was characterized by the presence of fine filaments, a finding consistent with fimbriae, that radiated from the surface of the cocci to the microvillus brush border, as well to adjacent bacteria (4, 6, 20). There was no evidence in the present or prior cases that adherence of enterococci results in microvillus effacement or cup and pedestal formation, as is characteristic of attaching and effacing *E. coli* (4, 6, 17, 20). Enterococci appear to be sufficient to mediate diarrhea in suckling foals, gnotobiotic piglets, and rat pups experimentally infected with *E. hirae* cultured from the feces of animals with enteroadherent bacteria and diarrhea (6–8, 22). However, the mechanisms by which enteroadherent enterococci cause diarrhea remain unclear. Villous architecture is invariably preserved, subepithelial inflammation is minimal in infected animals (4, 6–8, 13, 15, 17), and enterotoxin secretion has not been demonstrated (6, 22). Infected animals have significantly decreased small intestinal lactase and alkaline phosphatase activities, suggesting that the diarrhea may be malabsorptive (22). Little to no research aimed at further clarifying the pathogenesis of enterococcal diarrhea has been performed in the last 2 decades. Whether *E. hirae* was the primary or contributing cause of diarrhea, death, or euthanasia in the kittens reported here is unknown. The invasive potential of *E. hirae* is suggested by prior reports wherein cats with enteroadherent infection and diarrhea succumbed to acute death or terminal bacteremia and

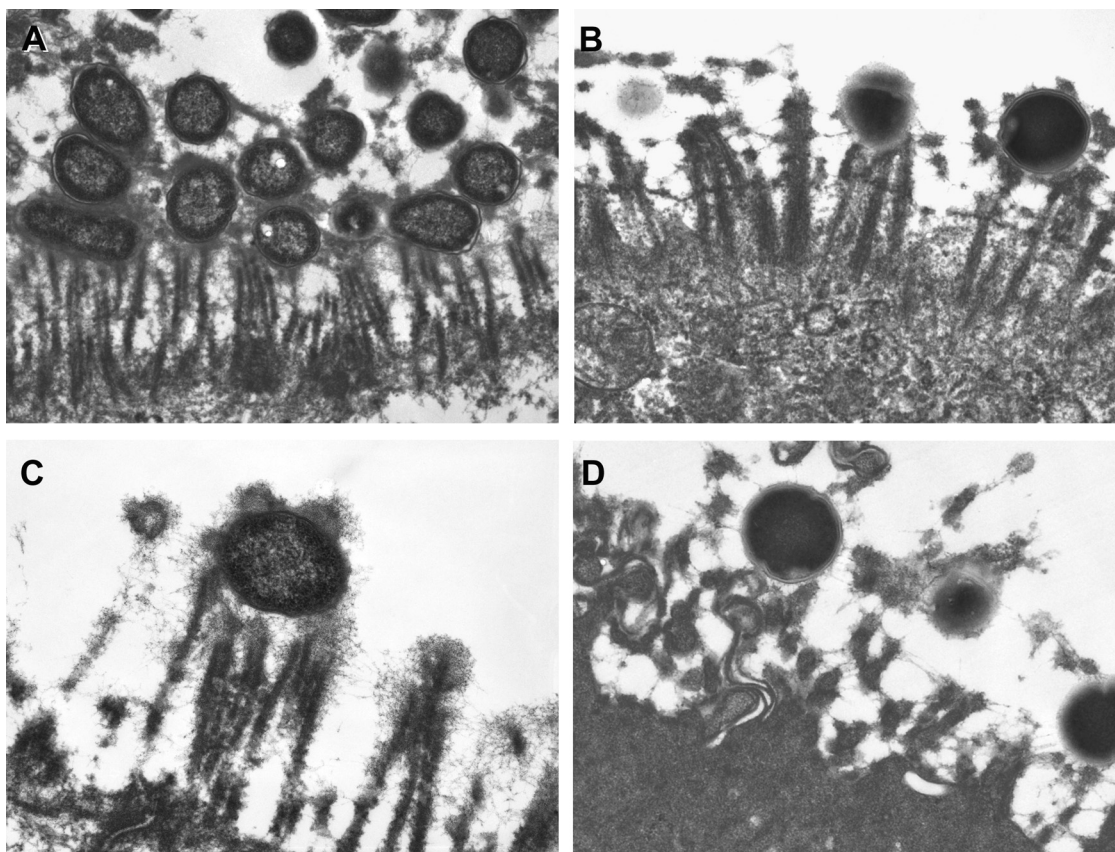


FIG. 4. Transmission electron micrographs of feline small intestinal epithelium from kittens naturally infected with enteropathogenic *E. coli* (A and C) or enteroadherent *E. hirae* (B and D). Interactions between the bacteria and the microvillus brush border of the epithelial cells are similar in both infections.

septic shock (13, 17). In dog pups with enteroadherent *E. hirae* infection, cocci were observed within intestinal epithelial cell lysosomes (4, 6). Commonalities between the kittens reported here and prior descriptions of enteroadherent *Enterococcus* infection suggest that young, suckling animals are predisposed to conditions capable of promoting pathogenicity of these normally commensal bacteria (3, 4, 6–8, 17, 20, 22, 24). These conditions could include immaturity of immune function or failure of passive transfer, impaired colonization resistance, stressful housing conditions, concurrent infectious disease as identified in several of the cats reported here, or antibiotic administration.

Although the prevalence of enteroadherent *E. hirae* infection in pre-weaning-age kittens is unknown, kitten mortality in association with diarrhea is common in shelter and rescue facilities, and necropsy examinations are seldom requested. Based on results of the present study, it appears that *E. hirae* may be a common cause of enteroadherent bacterial infection in kittens and easily misdiagnosed as *E. coli* if based on light microscopic examination alone. Consequently, we advocate the routine use of a Gram stain as the first discriminatory test to diagnosis of enteroadherent bacterial infection in kittens.

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