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. Gookin1*

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 containing 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 \textit{E. coli} infection was pre-
sumed. 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 kit-
ten). Aerobic bacterial culture of feces was performed at the time of necropsy 
for four kittens, three of which were positive for \textit{E. coli}. Genomic DNA extracted 
from selected \textit{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 \textit{E. coli}, one kitten with CNF-1 and CNF-2-positive beta-hemolytic \textit{E. coli}, and one kitten was culture-negative for \textit{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 
testinal tract disease.

\textbf{FISH.} Formalin-fixed, paraffin-embedded tissue samples were sectioned at 
a thickness of 4 \mu m and mounted on poly-L-lysine-coated slides. The tissue sec-
tions were deparaffinized, rehydrated, and air dried prior to hybridization with 
fluorescein in situ hybridization (FISH) probes at a working strength of 5 ng/\mu 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 \textit{E. coli}/Shigella (14) or \textit{Enterococcus} spp. (Enc221, 5’-Cy-CAC 
CGGGGTCCATCCATCA-3’) were simultaneously applied. The genus-spe-
cific probe for enterococci, probe Enc221 has been demonstrated to have an 
specificity of 100% when tested against 14 different \textit{Enterococcus} species and 19 
nonenterococcal reference strains (25). Probe specificity was additionally 
evaluated by using a sense probe non-Enc221 (5’-6FAM-TGATGGATGGACCCCG 
GGTG-3’) and by including positive and negative control slides in each assay. 
Positive control slides included formalin-fixed, paraffin-embedded intestinal tis-
sue from a pig and dog with culture and multiple PCR-confirmed eae-positive, 
enteropathogenic \textit{E. coli} infection and by using \textit{Enterococcus} as a negative control. 
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 
opacification 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).

\textbf{DNA extraction from formalin-fixed, paraffin-embedded intestinal tissue.} 
Intestinal tissue (\sim 80 mg), corresponding in location to the site of microscopically 
observed enterohedent bacterial infection, was excised from the paraffin block of 
each infected and all control kittens. Between each tissue block, an equivalent amount 
of intestinal tissue (\sim 80 mg) was excised from the paraffin block of each 
test kitten. Intestinal tissue samples obtained from seven age-matched control 
kittens, euthanized by a local animal control facility for reasons unrelated to 
testinal disease. All tissue samples were subjected to \textit{Enterohedent \textit{E. coli} infection and in control kittens.} In the small intes-
tines of all seven kittens extensive colonies of cocciocbacilli were present in diplococcol and palisading formations over the lu-
minal surface (Fig. 1). In one kitten the colon was also in-
volved. 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, hypereosino-
philic, and often contained pyknotic nuclei. Within the super-
ficial and deep lamina propria only occasional neutrophils and 
small numbers of lymphocytes were observed. In three kittens 
concurrent intestinal pathogens were identified, including 
\textit{Tritrichomonas foetus}, panleukopenia virus, and adenovirus (1 kitten each). 
Enterohedent bacteria were not observed in intestinal tissue samples obtained from seven age-matched 
control kittens euthanized for reasons unrelated to gastroin-
testinal disease.

\textbf{FISH using eubacterial and \textit{E. coli}/Shigella-specific oligonu-
cleotide probes.} Because each kitten had been presumptively 
diagnosed with enterohedent \textit{E. coli} infection on the basis of 
light microscopy, \textit{in situ} hybridization was initially per-
formed using individual and simultaneously applied eubac-
terial (Eub338) and \textit{E. coli}/Shigella-specific oligonucleotide probes. Hybridization of both probes to the enterohedent 
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 \textit{E. coli} infection. For the remain-
ing five kittens, neither probe hybridized to the enterohedent 
bacteria. \textit{E. coli} was also not observed by FISH in small intes-
tinal tissues from age-matched control kittens, with the excep-
tion of one case in which a few \textit{E. coli} organisms were observed in 
the intestinal lumen.

\textbf{RESULTS}

Light microscopic findings in kittens with enterohedent 
\textit{E. coli} infection and in control kittens. In the small intes-
tines of all seven kittens extensive colonies of cocciocbacilli were present in diplococcol and palisading formations over the lumen (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, hypereosinophilic, 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 \textit{Tritrichomonas foetus}, panleukopenia virus, and adenovirus (1 kitten each). Enterohedent bacteria were not observed in intestinal tissue samples obtained from seven age-matched control kittens euthanized for reasons unrelated to gastrointestinal disease.

\textbf{FISH using eubacterial and \textit{E. coli}/Shigella-specific oligonucleotide probes.} Because each kitten had been presumptively diagnosed with enterohedent \textit{E. coli} infection on the basis of light microscopy, \textit{in situ} hybridization was initially performed using individual and simultaneously applied eubacterial (Eub338) and \textit{E. coli}/Shigella-specific oligonucleotide probes. Hybridization of both probes to the enterohedent 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 \textit{E. coli} infection. For the remaining five kittens, neither probe hybridized to the enterohedent bacteria. \textit{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 \textit{E. coli} organisms were observed in the intestinal lumen.
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 \textit{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 \textit{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 \textit{Enterococcus} sp.-specific oligonucleotide probes after pretreatment of tissue sections with lysozyme. Hybridization of both eubacterial and \textit{Enterococcus} probes were observed in the five kittens with enteroadherent Gram-positive bacterial infection (Fig. 3) and in intestinal tissue from a pig with \textit{E. durans} infection. In the two kittens with enteroadherent Gram-negative \textit{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 \textit{Enterococcus} probe or the \textit{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 \textit{E. hirae}. Both \textit{E. hirae} and \textit{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 \textit{E. durans}- and \textit{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 \textit{E. hirae} muramidase gene. Sequence analysis of the PCR amplicons revealed 99\% sequence identity to that of \textit{E. hirae} (accession no. [2816] NICKLAS ET AL. J. CLIN. MICROBIOL. on August 15, 2017 by guest http://jcm.asm.org/ Downloaded from).
Amplification of products corresponding in size to the target sequence of the *E. durans* muramidase gene homolog (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.

**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. 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.](http://jcm.asm.org/Downloaded from)
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 necrotoxigenic 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 eubacteria-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 necrotoxigenic 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. 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.
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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REFERENCES


