Mortality in kittens is associated with a shift in ileum mucosa-associated enteroccoci from E. hirae to biofilm-forming E. faecalis and adherent E. coli

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Mortality in Kittens is Associated with a Shift in Ileum Mucosa-Associated Enteroccoci from *E. hirae* to Biofilm-Forming *E. faecalis* and Adherent *E. coli*.

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Abstract

Approximately ~15% of foster kittens die before 8-wks of age with most of these kittens demonstrating clinical signs or post-mortem evidence of enteritis. While a specific cause of enteritis is not determined in most cases; these kittens are often empirically administered probiotics that contain enterococci. The enterococci are members of the commensal intestinal microbiota but can also function as opportunistic pathogens. Given the complicated role of enterococci in health and disease, it would be valuable to better understand what constitutes a "healthy" enterococcal community in these kittens and how this microbiota is impacted by severe illness. In this study, we characterize the ileum mucosa-associated enterococcal community of 50 apparently healthy and 50 terminally ill foster kittens. In healthy kittens, *E. hirae* was the most common species of ileum mucosa-associated enterococci and was often observed to adhere extensively to the small intestinal epithelium. These *E. hirae* isolates generally lacked virulence traits. In contrast, non-*E. hirae* enterococci, notably *E. faecalis,* were more commonly isolated from the ileum mucosa of kittens with terminal illness. Isolates of *E. faecalis* had numerous virulence traits and multiple antimicrobial resistance. Moreover, attachment of *E. coli* to the intestinal epithelium was significantly associated with terminal illness and was not observed in any kitten with adherent *E. hirae*. These findings identify a significant difference in species of enterococci cultured from the ileum mucosa of kittens with terminal illness compared to healthy kittens. In contrast to prior case studies that associate enteroadherent *E. hirae* with diarrhea in young animals, these controlled studies identified *E. hirae* as more often isolated from healthy kittens and adherence of *E. hirae* as more common and extensive in healthy compared to sick kittens.

Introduction

An estimated 74 million owned (1) and ~70 million feral (2) cats currently reside in the United States. Population projections estimate that these cats give birth to roughly 180 million kittens per year (3). Each year inestimable numbers of kittens are abandoned, orphaned, or relinquished shortly after birth to be fostered by an 4,000 to 6,000 U.S. animal shelters (4). While the exact statistics are unknown, ~15% of kittens fostered by these shelters die or are euthanized because of illness before they reach 8-weeks of age (5-9). An obvious cause of illness is unknown in as many as 20% of kittens (6); however, the majority are reported to have clinical signs of diarrhea (6, 10) or post-mortem evidence of enteritis at the time of death (11).

Many infectious agents are known or suspected causes of gastrointestinal morbidity in young kittens. Bacterial culprits however, are difficult to decipher as they reside amongst a large and diverse enteric microbiome. The Gram-positive enterococci are an important part of the enteric microbiome and are generally considered to be gastrointestinal commensals. *Enterococcus faecium* in particular is commonly administered as a probiotic to kittens having diarrhea (12, 13). However, several other strains of *E. faecium* and *E. faecalis* are recognized as serious potential pathogens. The clinical importance of these enterococci is largely attributed to their ability to 1) acquire multiple antimicrobial resistance (14); 2) opportunistically infect tissues outside of the gastrointestinal tract (15-17); and 3) to form resilient environmental biofilms (18, 19).

While the clinical significance of enterococci in extra-gastrointestinal and nosocomial infections are well recognized, little is known about whether enterococci also play a significant role in the pathogenesis of disease inside the gastrointestinal tract. In recent years certain enterococci, most notably *E. hirae* and *E. durans*, have been observed in numerous different

species of neonatal animals to intimately and extensively colonize the mucosal surface of the small intestine in a manner similar to that of enteropathogenic *Escherichia coli* (EPEC) (20-28). While most of these reports describe animals having concurrent signs of diarrhea, the association of enteroadherent enterococci with clinical disease of the gastrointestinal tract in these young animals remains unclear.

Given the conflicting roles of enterococci as both members of the commensal microbiota and opportunistic pathogens, a better understanding of what constitutes the "healthy" enterococcal community in very young kittens and how this microbiota is impacted by severe illness in this population is needed. This is particularly true with regard to the small intestine, where commensal microbial-intestinal epithelial cell interactions can have a profound impact on gastrointestinal function, colonization resistance, and presumably neonatal survival. Consequently, the purpose of the present study was to determine the prevalence, species diversity, virulence traits, clonality, and antibiotic resistance of the mucosa-associated enterococcal community of the small intestine of very young kittens and their association with disease mortality. In addition, the prevalence and identity of enteroadherent bacteria in the small intestine of these kittens was determined *in situ*.

MATERIALS AND METHODS

Study populations. One hundred unrelated kittens ≤ 12-weeks of age were obtained from two collaborating facilities over a period of 18-months. Fifty kittens were in apparent good health and euthanized at a local animal control facility due to overpopulation (Group A). Fifty kittens had died or were euthanized due to severe illness while under foster care at a local county Society for Protection and Care of Animals (SPCA) (Group B). No kittens were euthanized for

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the purpose of this study. Demographic information was obtained for each kitten and pre-mortem clinical signs were used to categorize Group B kittens as having primarily gastrointestinal, respiratory, or other/unknown underlying disease. Immediately post-mortem kittens were kept refrigerated at 4°C and later transported on ice packs from each collaborating facility to North Carolina State University College of Veterinary Medicine for procurement of study samples.

Light microscopic examination for enteroadherent bacteria and gastrointestinal pathology. A single full-thickness sample of the stomach, duodenum, ileum, and colon were obtained from each kitten and fixed in 10% neutral buffered formalin for a period of ≥ 24 hours prior to processing into paraffin. Paraffin embedded tissues were sectioned at 5 μm thickness and stained with hematoxylin and eosin and Gram stains using routine methods. All tissues from each kitten were examined independently by means of light microscopy by two study investigators (PM, JLG). Each investigator, blinded to the origin and disease state of the kittens, assessed samples for the presence of colonies of bacteria that were intimately associated with the brush border of the intestinal epithelium as has been described for all prior reports of enteroadherent enterococcal infections (20-28). This characteristic light microscopy finding has been shown in representative specimens to correlate with the observation of filamentous projections between the enterococci and epithelial microvilli using transmission electron microscopy (24-26, 28). The final determination of enteroadherent bacterial infection in each sample was reached by consensus. All tissues from each kitten were additionally examined by a board certified veterinary pathologist (PM) for the presence of histopathogical lesions consistent with gastrointestinal disease.

Fluorescence *in situ* hybridization. Formalin-fixed, paraffin-embedded tissue samples from kittens with light microscopic evidence of enteroadherent bacteria were sectioned at a

thickness of 4 µm, mounted on poly-L-lysine coated slides and processed for fluorescence *in-situ* hybridization (FISH) as previously described in detail (28, 29). Hybridizations were performed using the universal eubacterial probe Eub338 (29), labeled at the 3′ end with 6-FAM, and subsequently examined using specific probes directed against *E. coli/Shigella* (5′-Cy3-GCAAAGGTATTAACTTTACTCCC-3′) (29) or *Enterococcus* spp. (Enc221 5′-Cy3-CACCGCGGGTCCATCCATCA-3′) (30) at working strengths of 5 ng/µl (29) as previously described (28, 29). Positive control slides for *E. coli* included formalin-fixed, paraffin-embedded intestinal tissue from a pig and a dog that were diagnosed with enteropathogenic *E. coli* based on light microscopic observation of palisades of Gram negative bacteria adhering to the intestinal epithelium, positive fecal culture for *E. coli*, and PCR amplification of the enterocyte attaching and effacing gene (*eae*). Positive control slides for *Enterococcus spp.* were formalin-fixed, paraffin-embedded intestinal tissue samples from a pig that was diagnosed with enteroadherent *Enterococcus spp.* infection based on light microscopic observation of palisades of Gram positive bacteria extensively colonizing the mucosal surface as previously described in this species (22, 23). For each kitten identified with enteroadherent enterococci, the enterococci were semi-quantitatively described on the basis of the number of bacteria present (scant, mild, moderate, severe) and extent of colonization of the epithelium (focal, diffuse).

DNA extractions from formalin-fixed, paraffin-embedded intestinal tissue. In order to identify the species of adherent enterococci as observed by light microscopy and FISH, DNA was extracted from each corresponding paraffin-embedded tissue block. Fifteen 5 µm serial sections of each block were microwaved at 5-sec intervals in 200 µl ATL buffer (Qiagen, Valencia, CA) until the paraffin liquefied. The solution was centrifuged (150 × g for 10-min) followed by removal of the paraffin ring using a sterile pipette tip. DNA extraction from the

remaining solution was performed as previously described (28). 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). 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.

PCR identification of enteroadherent enterococci and enteropathogenic *E. coli*. 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 approximately 400 b.p. gene sequence of feline GAPDH DNA as previously described (31). Subsequent PCR was performed using species-specific primer pairs for *mur-2* (*E. hirae*) (32), *ddl* (*E. faecium* and *E. faecalis*) (33-35), and *eae* (EPEC) (36) using previously published reaction conditions (33-36). Amplicons were visualized by UV illumination after electrophoresis of 10 μl of the reaction solution in a 1.5% agarose gel containing ethidium bromide. Identity of each reaction product was confirmed by sequence analysis (GENEWIZ Inc. Research Triangle Park, NC). Positive controls included *E. hirae* (ATCC 8043), *E. durans* (ATCC 6056), *E. faecalis* and *E. faecium* (a gift from the Clinical Microbiology Laboratory at North Carolina State University) and *eae*-positive *E. coli* (a gift from Dr. Karen Post, North Carolina Department of Agriculture and Consumer Services Veterinary Diagnostic Laboratory System).

Transmission electron microscopy. Intestinal tissue (~80 mg), corresponding in location to the site of microscopically-observed enteroadherent *E. hirae*, was cut from the original paraffin block of two kittens and processed for transmission electron microscopy as previously described (28).

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Isolation and identification of enterococci. During necropsy of each kitten, the distal ileum was opened longitudinally and an area of the surface mucosa devoid of visual debris was rubbed with a sterile cotton tipped applicator prior to streaking onto Columbia agar with colistin and naladixic acid (CNA) containing 5% sheep blood (BD Diagnostic Systems, Sparks, MD). Individual catalase-negative colonies (range 1 to ≥ 10 per kitten) were sub-cultured onto trypticase soy agar containing 5% sheep blood (BD Diagnostic Systems, Sparks, MD). Isolates were frozen in 2× brain heart infusion broth plus 50% glycerol prior to shipment on dry ice to Kansas State University. Isolates were subsequently selected on mEnterococcus agar (Difco, BD Diagnostic Systems, Sparks, MD) and confirmed at the genus level by the esculin hydrolysis test using Enterococcosel broth (Difco, BD Diagnostic Systems, Sparks, MD) incubated at 44.5°C for 24 h (37). Multiplex PCR was used to identify four common species, *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* (33). *Enterococcus faecalis* ATCC 19433, *E. faecium* ATCC 19434, *E. casseliflavus* ATCC25788, and *E. gallinarum* ATCC49579 were used as positive controls. PCR amplification and sequencing of the manganese-dependent superoxide dismutase gene (*sodA*) was carried out for isolates that were not identified by multiplex PCR (38).

Screening for virulence traits by genotype and phenotype. Multiplex PCR was performed to screen the identified isolates for four putative virulence determinants (*gelE*, gelatinase; *cylA,* cytolysin; *asa1,* aggregation substance; and *esp,* enterococcal surface protein) (39). In addition, *E. hirae*-specific primer sets for the virulence genes *gelE*, *cylA,* and *asa1* were used to screen a subset of 60 *E. hirae* isolates from healthy kittens and 51 *E. hirae* isolates from sick kittens (40). *E. faecalis* MMH 594 and *E. hirae* AA-1c and Aa-3B were used as positive controls. Gelatinase activity was tested on Todd Hewitt Agar (Difco, BD Diagnostic Systems, Sparks, MD) supplemented with 1.5% skim milk (41). All identified isolates were spotted and

after 16-20 h of incubation at 37°C were examined for a clearance zone surrounding the colonies (42). *Enterococcus faecalis* OG1RF(pCF10) was used as a positive control. Polystyrene round-bottomed 96 well plates (Corning Inc., Corning, NY) were used to detect *in vitro* biofilm formation from identified isolates. The test strains were cultivated overnight in M17 broth (Difco, BD Diagnostic Systems, Sparks, MD) at 37°C. A ratio of 1:100 of the overnight culture was diluted in fresh M17 broth. Microtiter plates were incubated at 37°C without agitation for 24 h to allow for bacterial growth and biofilm formation (42). Biofilm formation was quantified using the crystal violet staining method as described by Hancock and Perego (43). All experiments included blank wells (medium without any inoculum), *E. faecalis* V583 (positive control for *gelE* and *sprE* expression and biofilm formation) and *E. faecalis* V583Δ*gelE* (negative control with isogenic deletion of *gelE* that does not form biofilm) and were replicated five times. Control strains (MMH 594, V583, OG1RF, and V583Δ*gelE*) were obtained from Dr. Lynn Hancock (Kansas State University). The deletion mutant strain V583Δ*gelE* was described by Thomas et al. (44). Control strains of *E. hirae* AA-1c and Aa-3B were obtained from our previous study by Ahmad et al. (45).

Pulsed-field gel electrophoresis (PFGE). Selected isolates of *E. hirae* and *E. faecalis* were genotyped by means of PFGE performed using *Sma*I according to a previously published protocol (46) that was modified by replacing lysostaphin with mutanolysin (final concentration, 400 U/ml) in the bacterial digest. For accurate comparison of gel images, H9812 strain of *Salmonella* serotype Braenderup (ATCC BAA-664) digested with *Xba*I was included in 3 lanes of each gel. A position tolerance of 1% was used for band matching. Using Bionumerics 4.6 software (Applied Maths, Austin, TX, USA), dendrograms were created using a similarity matrix

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of Dice coefficients and the un-weighted pair group method with arithmetic mean (UPGMA) algorithm.

Antimicrobial susceptibility testing. Minimum inhibitory concentrations (MIC) for selected isolates of *E. hirae* and *E. faecalis* were determined by the broth dilution method using Mueller-Hinton broth, overnight incubation at 37°C in 5% CO_2 and a commercially available MIC plate according to the manufacturer's instructions (Gram Positive National Antimicrobial Resistance Monitoring System Plate [CMV3AGPF], Trek Diagnostic Systems, Cleveland, OH, USA). MICs were interpreted as either susceptible or non-susceptible using Clinical Laboratory Standards Institute (CLSI) guidelines (47) when available; otherwise NARMS (46) breakpoints were used. Antimicrobials included on the plate were: tigecycline (TGC), tetracycline (TET), chloramphenicol (CHL), daptomycin (DAP), streptomycin (STR), tylosin tartrate (TYLT), quinupristin/dalfopristin (SYN), linezolid (LZD), nitrofurantoin (NIT), penicillin (PEN), kanamycin (KAN), erythromycin (ERY), ciprofloxacin (CIP), vancomycin (VAN), lincomycin (LIN), and gentamicin (GEN).

Statistical analysis. Data were tested for significant differences in distribution of observations between groups or culture isolates using Chi-square and Fisher exact tests. Differences in the mean value of continuous data between groups of kittens were analyzed using a Student's t-test. Analyses were conducted using commercial software (SigmaPlot 12, Systat Software Inc San Jose CA) and an assigned p-value of <0.05.

RESULTS

Kitten population demographics. One hundred kittens were included in this study (Table 1). Kittens that died or were euthanized because of severe illness (Group B) had a significantly

lower average body weight compared to that of apparently healthy kittens (Group A). Due to the untimely nature of death or euthanasia of kittens with severe illness, a significant difference in duration of time between death and necropsy was observed between the two groups.

Prevalence of clinical signs and light microscopic evidence of gastrointestinal pathology in healthy versus sick kittens. The most commonly reported pre-mortem clinical signs in Group B kittens were referable to gastrointestinal illness, mainly diarrhea. Clinical signs of illness were not reported pre-mortem for any Group A kittens (Table 2). Light microscopic evidence of gastrointestinal tract pathology and/or infectious agents were identified in both groups of kittens (Table 3). Lesions observed via light microscopy in the gastrointestinal tract of kittens were largely non-specific as to etiology and characterized in the many cases as consisting of mild inflammatory infiltrates and crypt abscesses (Supplementary Table 1).

Adherence of *Enterococcus* spp. to the small intestinal epithelium is more common and extensive in healthy versus sick kittens. A total of 9 Group A and 7 Group B kittens were observed to have Gram-positive bacteria adherent to the intestinal epithelium by means of light microscopy (Table 4). In each case the Gram-positive bacteria were identified as enterococci by means of positive hybridization to the *Enterococcus* spp.-specific probe Enc221 (Figure 1). For species identification of adherent enterococci, DNA was extracted from each paraffin-embedded intestinal specimen in which the adherent enterococci were observed. In 8/9 Group A kittens, *Enterococcus* species-specific PCR performed on the extracted DNA identified *E. hirae* and not *E. faecalis* or *E. faecium* as the adherent enterococci. In contrast, *E. hirae* was identified as the adherent *Enterococcus* sp. in only 1/7 Group B kittens. The remaining Group B kittens were PCR-negative for *E. hirae*, *E. faecalis* and *E. faecium* (Table 4). Feline GAPDH gene sequences were amplified from each extracted DNA sample.

The anatomic location, number and extent of enteroadherent enterococci present throughout the intestinal tract differed descriptively between Group A and Group B kittens (Table 3). In Group A kittens, adherence of enterococci was restricted to the small intestine and the bacteria were more often observed in large numbers that extensively colonized the surface epithelium. In Group B kittens, adherence of enterococci was also demonstrated in the colon and the bacteria were observed in scant numbers to focally colonize the epithelium (Table 5 and Figure 2). Using two representative specimens that were identified by light microscopy as having scant versus severe adherence of enterococci, TEM was utilized to confirm the ultra-structural presence of direct interaction between the enterococci and the intestinal epithelial microvilli (Figure 3). There were no associations between the presence of enteroadherent enterococci and light microscopic evidence of gastrointestinal inflammation, duration of time between death and necropsy, presence or absence of autolysis, or whether the kitten had died or was euthanized.

Adherence of *E. coli* to the small intestinal epithelium is associated with kitten mortality. No kittens in Group A and 9 kittens in Group B were observed to have palisades of Gram-negative bacteria adherent to the small intestinal (n=6) or colonic epithelium (n=3) by means of light microscopy (Table 4). In each kitten the Gram-negative bacteria were identified as *E. coli* by positive hybridization to the *E. coli/Shigella* spp.-specific oligonucleotide probe. In 3/9 Group B kittens, *eae* gene sequences were amplified from DNA extracted from the corresponding paraffin-embedded tissue specimen. Notably, enteroadherent enterococci were not observed in any Group B kittens diagnosed with adherent *E. coli* infection.

Ileum mucosa-associated enterococci are represented by *E. hirae* in apparently healthy kittens and *E. faecalis* in sick kittens. Selective culture of the ileum mucosa-associated bacteria for enterococci generated a total of 331 *Enterococcus* spp. isolates from 31 Group A and

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24 Group B kittens. Six different species of enterococci were represented. *E. hirae* was the most common species isolated from the kittens and was identified significantly more often in kittens from Group A. In contrast, *E. faecalis* was isolated significantly more often from kittens in Group B (Table 6). There were no significant differences in isolation of *E. hirae* in either group of kittens based on the duration of time between death and plating of intestinal swab samples. Sixteen kittens in Group B received oral antibiotics for an unspecified duration prior to death including amoxicillin (n=9), cefazolin (n=5), doxycycline (n=2), amoxicillin-clavulanate (n=1), and azithromycin (n=1). However, a history of antibiotic administration was not significantly associated with isolation of either *E. faecalis* or *E. hirae*. *Enterococcus faecium* was isolated from 3 kittens in Group B, one of which had a history of receiving an *E. faecium*-containing probiotic (FortiFlora, Nestlé Purina, Vevey Switzerland). The same probiotic was historically given to 6 additional Group B kittens, none of which had *E. faecium* isolated from the ileum mucosa. An unbiased representation of the diversity of isolates obtained from individual Group A and B kittens each having ≥ 8 isolates obtained is shown in Figure 4.

Ileum mucosa-associated *E. hirae* lack phenotypic and genotypic determinants of enterococcal virulence. To further examine the ileum mucosa-associated isolates of *E. hirae* for phenotypic differences that may account for enteroadherence *in vivo*, isolates were selected for determination of gelatinase activity and biofilm formation *in vitro*. A total of 89 isolates from 6 Group A and 3 Group B kittens each having ≥ 9 *E. hirae* isolates showed no evidence of enteroadherent enterococci infection *in vivo*. These isolates were compared to 83 isolates from 6 Group A and 4 Group B kittens for which *in vivo* enteroadherent enterococci or *E. coli* infection were observed. Irrespective of the presence of adherent enterococci or *E. coli in vivo*, all tested isolates of *E. hirae* cultured from the ileum mucosa of Group A and Group B kittens displayed

similar weak gelatinase activity and failure to form biofilm *in vitro* (Figure 5). Putative genetic determinants of virulence (*gelE*, *cylA*, *asa1*, or *esp*) were not identified in any isolates of *E. hirae* (n=172) obtained from the ileum mucosa of any Group A or Group B kittens.

Ileum mucosa-associated *E. faecalis* have gelatinase activity, strong biofilm formation *in vitro*, and carry virulence traits. Having demonstrated that non-*E. hirae* enterococci were isolated almost exclusively from diseased kittens, 81 non-*E. hirae* isolates that were cultured from the ileum mucosa of 12 Group B kittens were tested for gelatinase activity and biofilm formation *in vitro*. Isolates of *E. faecalis* (41/54) obtained from 6/8 Group B kittens demonstrated strong biofilm formation and/or gelatinase activity. Additionally, all *E. faecalis* isolates carried one or more putative virulence traits (Figure 5).

Pulsed-field gel electrophoresis supports genomic similarity among *E. hirae* isolates cultured from the ileum mucosa of kittens with enteroadherent enterococci. To examine the genomic similarity among ileum mucosa-associated *E. hirae* isolates and their relationship to health versus illness of the kittens or the presence of enteroadherent enterococci or *E. coli in vivo*, PFGE was performed. One (if only 1 isolate) or 2 (if ≥ 2) isolates were examined from each kitten that had *E. hirae* cultured from ileum mucosa and either enteroadherent enterococci or *E. coli* identified by FISH. Two isolates were examined from each kitten that had ≥ 10 isolates of *E. hirae* cultured from ileum mucosa and for which neither enteroadherent enterococci or *E. coli* were identified (Figure 6). There was no distinct difference in PFGE pattern observed between isolates from Group A compared to Group B kittens. However, kittens within each group frequently shared similar genotypes of *E. hirae* but none of these genotypes was shared by both groups. Accordingly, the *E. hirae* population appeared to be genotypically distinguishable among the healthy versus sick kittens. There were no distinct differences in PFGE patterns observed

between *E. hirae* isolates based on whether or not they were obtained from kittens that had enteroadherent enterococci observed *in vivo* (Figure 6).

Isolates of *E. faecalis* from the ileum mucosa of kittens are genomically diverse. To determine the genomic similarity among *E. faecalis* isolates, as predominantly obtained from kittens in Group B, PFGE was performed on 1 or 2 representative isolates from each kitten (Figure 7). No clusters could be assigned to the *E. faecalis* isolates typed. Using a > 85% cut off value of similarity index, the isolates appeared quite diverse among different kittens. However, multiple isolates from the same kitten were genotypically indistinguishable indicating unique pulsotypes for each kitten.

Multiple and specific antimicrobial resistance is more common among isolates of *E. faecalis* compared to *E. hirae*. Antimicrobial susceptibility testing was performed on isolates of *E. faecalis* (n=18) and *E. hirae* (n=27), selected on the basis of different PFGE profiles, from 12 and 26 kittens respectively. Resistance to multiple ($\geq$ 3) antimicrobial drugs was significantly more common among the isolates of *E. faecalis* (17/18) compared to *E. hirae* (16/27)(* P=0.014, Fischer's exact test). The prevalence of resistance to specific antimicrobial drugs among kittens harboring *E. hirae* or *E. faecalis* are shown in Figure 8. Compared to *E. hirae*, *E. faecalis* were significantly more likely to be resistant to chloramphenicol, quinupristin/dalfopristin, erythromycin, lincomycin and ciprofloxacin (Supplemental Table 2). There was no significant relationship between multiple antimicrobial resistance and kitten group (A or B), PFGE pulsotype, or history of antibiotic administration.

DISCUSSION

This study is the first to characterize the enterococcal community of the distal small intestine of cats. We specifically focused on the mucosa-associated enterococci in the ileum of very young kittens. This decision was based on a strong association of small intestinal disease with mortality in this population, recognition that overall numbers of bacteria are highest in the ileum compared to other regions of the small intestine, importance of the ileum as a portal of entry for invasive bacterial pathogens, and likelihood of also observing concurrent adherent enterococci in this region. Moreover, bacteria that associate with the mucosal surface have a unique composition and influence on intestinal epithelial function as compared to those generally residing in the lumen (49). In fact, 60% of the terminally ill kittens in this study were euthanized or died with clinical signs and/or histopathological evidence of gastrointestinal tract disease; the majority of which was attributed to the small intestine. It was not the purpose of this study to establish the cause of death in these kittens and light microscopic examination of the intestinal tract was generally unrewarding in identifying any specific diagnoses.

This study identifies *E. hirae* as the dominant species of enterococci to colonize the ileal mucosa in apparently healthy young kittens. The only prior studies to examine the microbiota of the feline small intestine focused on the lumen-dwelling bacteria and were performed in older cats. In these studies, enterococci were either not identified in the small intestine (50) or were identified as represented predominantly by *E. faecalis* (51-53). *Enterococcus faecalis* (54-57), followed by *E. faecium* (58) and *E. hirae* (14), are the most commonly reported species of enterococci to dominate the fecal flora of cats, where the numbers of enterococci can average 10^6 colony forming units per gram of feces (14).

In kittens that died or were euthanized due to severe illness, there was a major difference in species of enterococci associated with the ileum mucosa. Significantly greater numbers of

these kittens were colonized by *E. faecalis* suggesting that the ileum mucosa-associated microbiota in sick kittens reflects a more adult fecal-like composition (54-57) of enterococci. Moreover, the *E. faecalis* isolates obtained from these kittens were characterized as carrying multiple genotypic and phenotypic attributes of virulence. This is in contrast to *E. faecalis* isolates from healthy cats where the carriage of virulence genes appears to be uncommon (59), albeit there are few reports examining genotypic virulence of *E. faecalis* in healthy cats (14, 60). Most notable among the virulence traits of *E. faecalis* from sick kittens was a high prevalence of *gelE*, a gene encoding the zinc metalloproteinase gelatinase, and concurrent documentation of gelatinase activity. Gelatinase is implicated in enhancing enterococcal virulence by conferring an ability to form solid-surface environmental biofilms (43) and in the present study *E. faecalis* gelatinase activity was associated with strong biofilm formation on polystyrene. There was additionally a high prevalence of *asa1*, a gene encoding for aggregation substance, among *E. faecalis* isolates from sick kittens. Aggregation substance is a surface bacterial adhesin that has been demonstrated to increase attachment of *E. faecalis* to intestinal epithelial cells *in vitro* (61). Whether *gelE* or *asa1* conferred to *E. faecalis* an ability to outcompete *E. hirae* for representation within the ileum mucosa-associated microbiota in these sick kittens is unknown.

In addition to carrying multiple genetic virulence factors, *E. faecalis* isolates were frequently resistant to tetracycline, erythromycin, lincomycin, quinupristin/dalfopristin, chloramphenicol, linezolid, and ciprofloxacin. None of the *E. faecalis* isolates demonstrated resistance to penicillin, aminoglycosides or vancomycin. Intrinsic or acquired resistance to quinupristin/dalfopristin, tetracyclines, and macrolides is commonly reported amongst feline isolates of *E. faecalis* (14, 55-57). In contrast, resistance to newer generation antimicrobial drugs (linezolid) or those commonly reserved for treatment of multi-drug resistant bacterial infections

in cats (ciprofloxacin) was somewhat surprising (14, 55). None of the kittens had a history of treatment with fluoroquinolones. Compared to *E. faecalis*, isolates of *E. hirae* obtained from apparently healthy or sick kittens were significantly less often resistant to multiple ($\geq$ 3) antimicrobial drugs. Less frequent antimicrobial resistance among *E. hirae* compared to *E. faecalis* and *E. faecium* of fecal-origin in cats has been previously documented (14).

Whether or not the virulent, antimicrobial-resistant isolates of *E. faecalis* colonizing the ileum mucosa of sick kittens in this study originated from commensal *E. faecalis* or were acquired from the environment is not known. Based on the PFGE genotypes of representative *E. faecalis* isolates from each kitten, multi-drug resistant strains did not share colonization of the ileum mucosa with other strains. Sick kittens possibly had a greater susceptibility and opportunity for colonization by resistant strains of *E. faecalis* as they spent time in a foster care or hospital environment in contrast to the apparently healthy kittens who were frequently euthanized shortly after their receipt to the animal control facility. An opportunistic infection of the sick kittens by virulent *E. faecalis* is supported by studies demonstrating frequent antibiotic resistance in fecal enterococci in cats from catteries, hospitalized cats, or resident cats in veterinary clinics (14, 56, 57). However, whether the colonization of ileum mucosa-associated microbiota by *E. faecalis* was a contributing cause or consequence of gastrointestinal disease and terminal illness in the sick kittens of this report is unknown. What appears more certain is that the intestinal enterococci of sick kittens may serve as a reservoir for potential transmission of antimicrobial resistance genes to the environment and/or to other hosts.

Apart from being identified as the dominant cultivable species of enterococci in the ileum mucosa-associated microbiota in apparently healthy young kittens, overt and extensive adhesion of *E. hirae* to the small intestinal epithelium was observed in 16% of the kittens in this

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population. Given that detection of this event requires light microscopy and only two biopsies of the small intestine from each kitten were examined, it is likely that the prevalence of this phenomenon was grossly underestimated. While adherent enterococci were also detected in the sick kittens, considerably fewer bacteria were observed and they were infrequently identified as to species by means of PCR. We attribute our inability to identify the adherent enterococci in these kittens as due to the presence of fewer bacteria (and less enterococcal DNA) and as less likely due to adhesion by non-*E. hirae* enterococci. Our finding that enteroadherent *E. hirae* is common and extensive in apparently healthy kittens provides a contrast to the numerous uncontrolled case reports describing enteroadherent enterococci in association with diarrhea in young animals (20-28). Although the identity and virulence attributes of the enteroadherent enterococci in most of these reports was not determined, it should be considered that the adherent enterococci may not have been the primary cause of diarrhea in these animals.

The mechanism(s) by which *E. hirae* adhere to the intestinal epithelium in these young kittens is not clear. Based on a comparative lack of virulence among the *E. hirae* isolates tested from the kittens, it is evident that the genotypic (*gelE*, *asa1*) and phenotypic (gelatinase activity) attributes generally regarded as essential for biofilm formation *in vitro* are not required for enteroadhesion *in vivo*. PFGE revealed that the *E. hirae* population is genotypically very diverse and clustering did not correlate with enteroadherence *in vivo*. A notable feature of enteroadherent *E. hirae* is an indistinguishable light microscopic appearance to that of EPEC that requires a Gram stain for their differentiation (28). It is likewise intriguing that adherence of *E. coli* was documented commonly and exclusively in sick kittens in this study but was not observed in any sick kitten with enteroadherent enterococci. This not only identifies enteroadherent *E. coli* as a

potentially important intestinal pathogen in young kittens, but also suggests that enteroadherence of *E. hirae* might competitively inhibit or otherwise deter the attachment of *E. coli*.

Results of this study identify *E. hirae* as the most common mucosa-associated species of enterococci to inhabit the small intestine in apparently healthy young kittens. Furthermore, adherence of *E. hirae* to the small intestinal epithelium was common and extensive in this population. Isolates of *E. hirae* generally lacked phenotypic and genotypic determinants of virulence. In contrast, kittens that died or were euthanized due to severe illness were significantly more often identified as colonized by *E. faecalis*. This population of *E. faecalis* was characterized by a high level of gelatinase activity, strong biofilm formation on polystyrene, presence of virulence determinants and multiple antimicrobial resistance. Moreover, attachment of *E. coli* to the intestinal epithelium was exclusively and significantly associated with terminal illness and was not documented in any kitten for which enteroadherent *E. hirae* was observed. These findings identify a significant difference in the species of enterococci colonizing the ileum mucosa of healthy versus terminally ill young kittens and suggest that *E. hirae* represents an important commensal in this population. Given the significance of small intestinal disease as a cause of mortality in young kittens, these findings have important implications toward identifying species of enterococci for their potential to significantly impact the survival of very young kittens.

assistance. Transmission electron microscopy was performed in the Laboratory for Advance Electron and Light Optical Methods in the College of Veterinary Medicine, North Carolina State University.

TABLES

Table 1 Demographic description of 100 kittens included in this study.

Population Description	Group A	Group B
Age (weeks)	6.2 ± 3.6 (range, 0.5 – 12)	5.8 ± 2.4 (range, 0.4 – 10)
Sex	22 Male 26 Female 2 Undetermined	25 Male 23 Female 2 Undetermined
Body weight (grams)	529 ± 250 (range, 122 – 1013)	360 ± 162*** (range, 132 – 922)
Time from death to necropsy (hours)	2.9 ± 3.7 (range, 0.75 – 20)	8.6 ± 6.0*** (range, 1 – 18.75)

Group A consists of 50 kittens that were apparently healthy and euthanized by a local animal control facility because of overpopulation. Group B consists of 50 kittens that died (n=28) or were euthanized (n=22) due to severe illness while under foster care at a local SPCA. Numbers represent average ± standard deviation. ***P ≤ 0.001 Student's *t*-test.

Table 2   Categorization of clinical signs reported pre-mortem for 100 kittens with or without enteroadherent *Enterococcus* spp. or enteroadherent *E. coli*.

Pre-Mortem Clinical Signs	Group A # (%) of kittens	# of kittens with enteroadherent bacteria having respective clinical signs *Enterococcus*	# of kittens with enteroadherent bacteria having respective clinical signs *E. coli*	Group B # (%) of kittens	# of kittens with enteroadherent bacteria having respective clinical signs *Enterococcus*	# of kittens with enteroadherent bacteria having respective clinical signs *E. coli*
Gastrointestinal	0 (0)			25 (50)	2	5
Upper Respiratory/Ocular	0 (0)			20 (40)	1	3
Wounded or disabled	0 (0)			7 (14)	0	2
Failure to thrive	0 (0)			4 (8)	2	0
Unexpected death	0 (0)			3 (6)	1	0
None recorded	50 (100)	9	0	2 (4)	1	1
Total # of kittens	**50**	**9**	**0**	**50***	**7**	**9**

*Eleven kittens in Group B had more than one category of clinical signs reported pre-mortem.

Table 3 Numbers of kittens having histopathological lesions and/or infectious agents identified by light microscopy and FISH based on anatomical location.

Anatomical Location	Group A				Group B			
	# (%) kittens with abnormal histopathology	Infectious agents identified by light microscopy (# kittens)	# kittens with enteroadherent bacteria confirmed by FISH		# (%) kittens with abnormal histopathology	Infectious agents identified by light microscopy (# kittens)	# kittens with enteroadherent bacteria confirmed by FISH	
			Entero-coccus	E. coli			Entero-coccus	E. coli
Stomach	2 (4)	Helicobacter (2)	0	0	0 (0)	Sarcina (1)	0	0
Duodenum	4 (8)		3	0	1 (2)		1	0
Ileum	3 (6)	Coccidia (2) Spirochetes (2)	7	0	13 (26)	Panleukopenia susp. (3) Coccidia (2) Spirochetes (1) Trichomonads (1)	6	6*
Colon	3 (6)	Spirochetes (17) Trichomonads (1) Ascarid eggs (1)	0	0	10 (20)	Spirochetes (8) Trichomonads (1)	4	3
Total # (%) kittens	10/50 (20)	21/50 (42)	9/50 (18)	0/50 (0)	19/50 (38)	13/50 (26)	7/50 (14)	9/50 (18)
Lung	1 (2)				7 (14)	FHV-1 susp (1) Toxoplasma susp. (2)	NE	NE
Hepatobiliary	4 (8)				4 (8)	Toxoplasma susp. (1)	NE	NE
Pancreas	0				1 (2)	Toxocara (1)	NE	NE
Total # (%) kittens	5/50 (10)	0/50 (0)			11/50 (22)	4/50 (8)		

Kittens may have had abnormal histopathology and/or infectious agents identified in more than one anatomical location. * In two kittens the small intestinal location of enteroadherent bacteria could not be ascribed to duodenum versus ileum. NE = tissues not examined by means of FISH.

Table 4   Number of kittens identified with enteroadherent bacteria and subsequent species identification.

In-situ Identity of Enteroadherent Bacteria	No. (%) of cats with enteroadherent bacteria	
	Group A	**Group B**
Enterococcus spp.	9/50 (18%)	7/50 (14%)
E. hirae confirmed by PCR	8/9 (89%)	2/7 (29%)*
Species undetermined by PCR	1/9 (11%)	5/7 (71%)*
Escherichia coli	0/50 (0%)	9/50 (18%)**
eae positive by PCR		3/9 (33.3%)
eae undetermined by PCR		6/9 (66.7%)

Enteroadherent bacteria were detected by light microscopy and identified to genus by fluorescence in-situ hybridization and to species by polymerase chain reaction (PCR) performed on the same formalin-fixed, paraffin-embedded tissue specimen in which enteroadherent bacteria were observed. Kittens were apparently healthy (Group A, n=50) or had died or were euthanized due to severe illness (Group B, n=50). *P $\leq$ 0.05 Fischer's exact test.

Table 5   Semi-quantitative description of the number and distribution of adherent enterococci in the intestinal tract of kittens.

Number of adherent enterococci	Group A			Group B		
	# kittens	Bacterial Distribution		# kittens	Bacterial Distribution	
		Focal	Diffuse		Focal	Diffuse
Scant	1	1		4	4	
Mild	3	2	1	2	2	
Moderate	2	1	1	1		1
Severe	3		3	0		
Total	**9**	**4**	**5**	**7**	**6**	**1**

Adherent enterococci were identified by means of fluorescence in-situ hybridization. Kittens were apparently healthy (Group A, n=9) or had died or were euthanized due to severe illness (Group B, n=7). Representative images of each description can be seen in Figure 2.

Table 6   Species identification of enterococci isolated from the ileum mucosa of apparently

healthy kittens.

Species Identification	Group A (n=153 isolates)		Group B (n=178 isolates)	
	No. (%) of total cats	No. (%) of total isolates	No. (%) of total cats	No. (%) of total isolates
E. hirae	29 (58)	148 (97)	14 (28)**	75 (42)
E. faecalis	2 (4)	3 (2)	10 (20)*	64 (36)
E. faecium	1 (2)	1 (0.5)	3 (6)	12 (7)
E. avium	0 (0)	0 (0)	2 (4)	18 (10)
E. gallinarum	0 (0)	0 (0)	1 (2)	8 (4)
E. seriolicida	1 (2)	1 (0.5)	1 (2)	1 (1)
Total	31/50 (62)†	153/153 (100)	24/50 (48)†	178/178 (100)

†Several kittens had multiple species of enterococci isolated. Enterococcal species were identified by either multiplex PCR (33) or sequencing of the *sodA* gene (38). The number (%) of total isolates of each species depicted is biased by variation between kittens in the number of isolates chosen for purification. An unbiased representation of the distribution of isolates between Group A and B kittens is shown in Figure 3. *P<0.05, **P<0.01 Fischer's exact test.
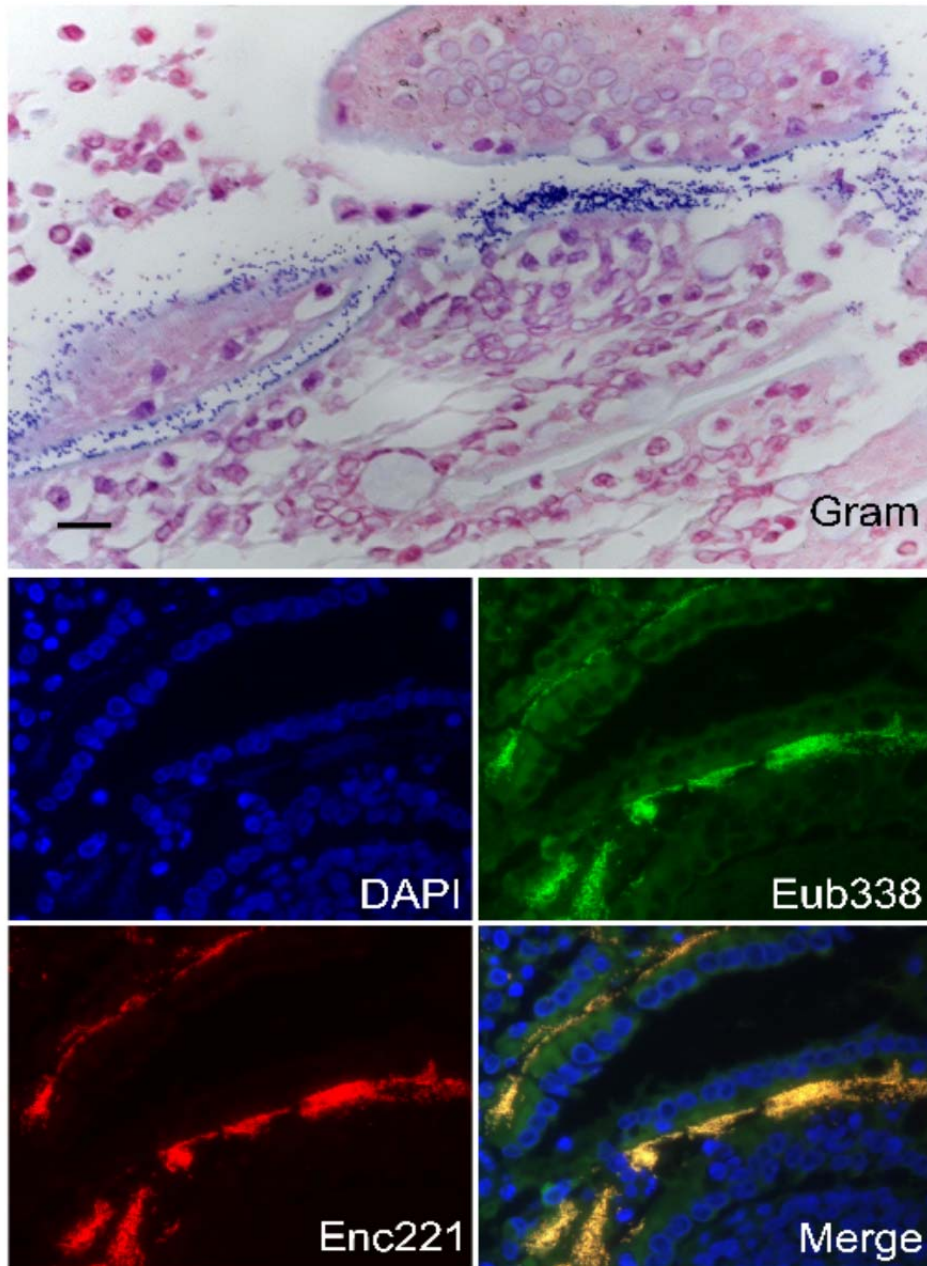
27

FIGURES

Figure 1   Demonstration of enterococci adhering to the small intestinal epithelium of an apparently healthy kitten. Extensive colonies of bacteria are demonstrated along the apical epithelium by means of Gram stain and fluorescence in situ hybridization using eubacterial (Eub-338-FAM) and *Enterococcus* spp.-specific (Enc-221-Cy3) oligonucleotide probes. Sections were nuclear counterstained with DAPI. Bar = 20 μm.
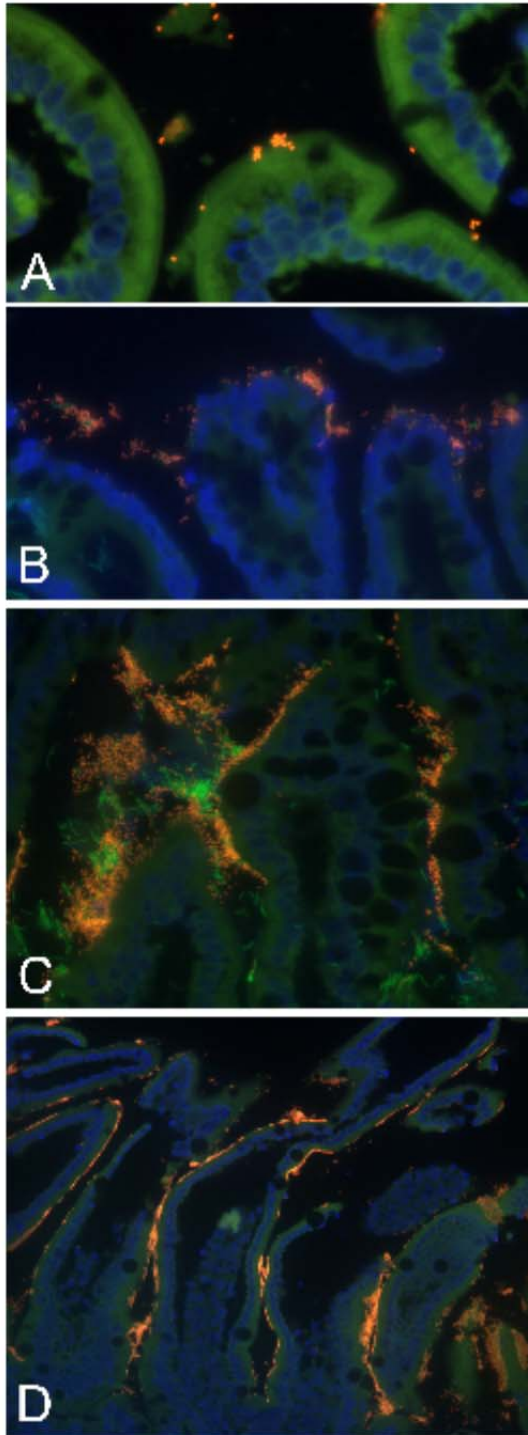
Figure 2   Representative results of semi-quantitative scoring of the number and extent of enteroadherent enterococci in 4 healthy Group A kittens. Panel A = scant, focal. Panel B = mild, focal. Panel C = moderate, diffuse. Panel D = severe, diffuse. Fluorescence in situ hybridization was performed using eubacterial (Eub-338-FAM) and *Enterococcus* spp.-specific (Enc-221-Cy3) oligonucleotide probes. Sections A, B, and D were nuclear counterstained with DAPI.
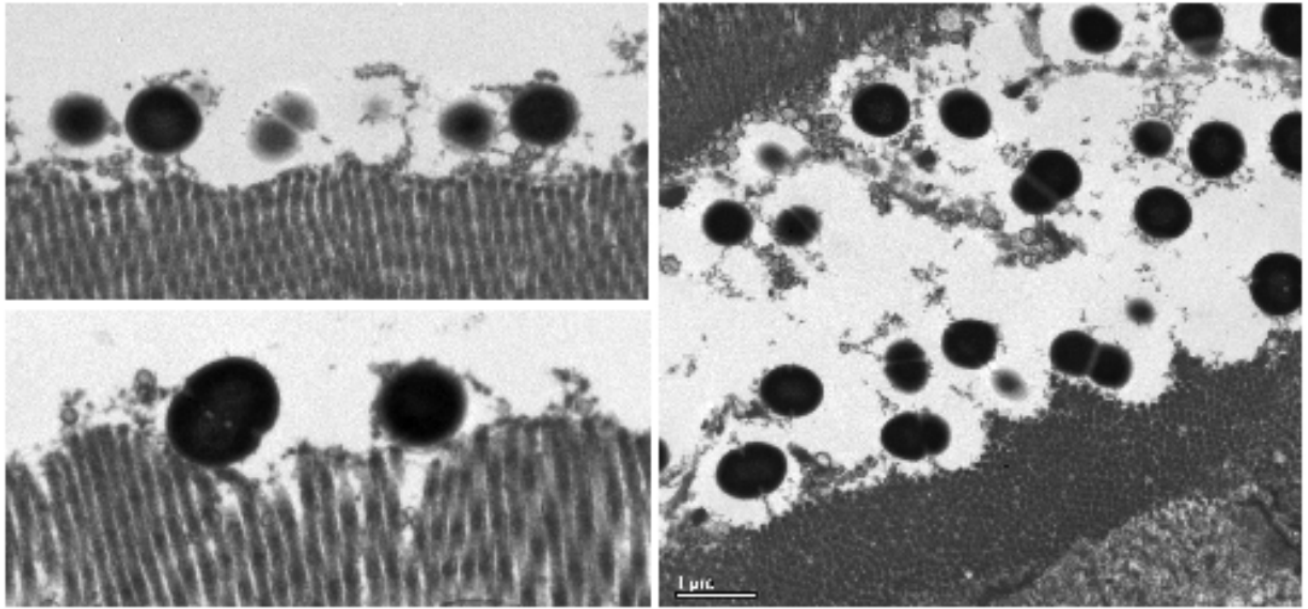
Figure 3 Transmission electron micrographs of enterococci interacting directly with the intestinal epithelial microvilli. The left panel micrograph was taken of a specimen with light microscopic evidence of scant, focal adherence of enterococci. The right panel micrograph was taken of a specimen with light microscopic evidence of severe diffuse adherence of enterococci. These specimens share the same origin as those shown in Figure 2 panel A and D, respectively.
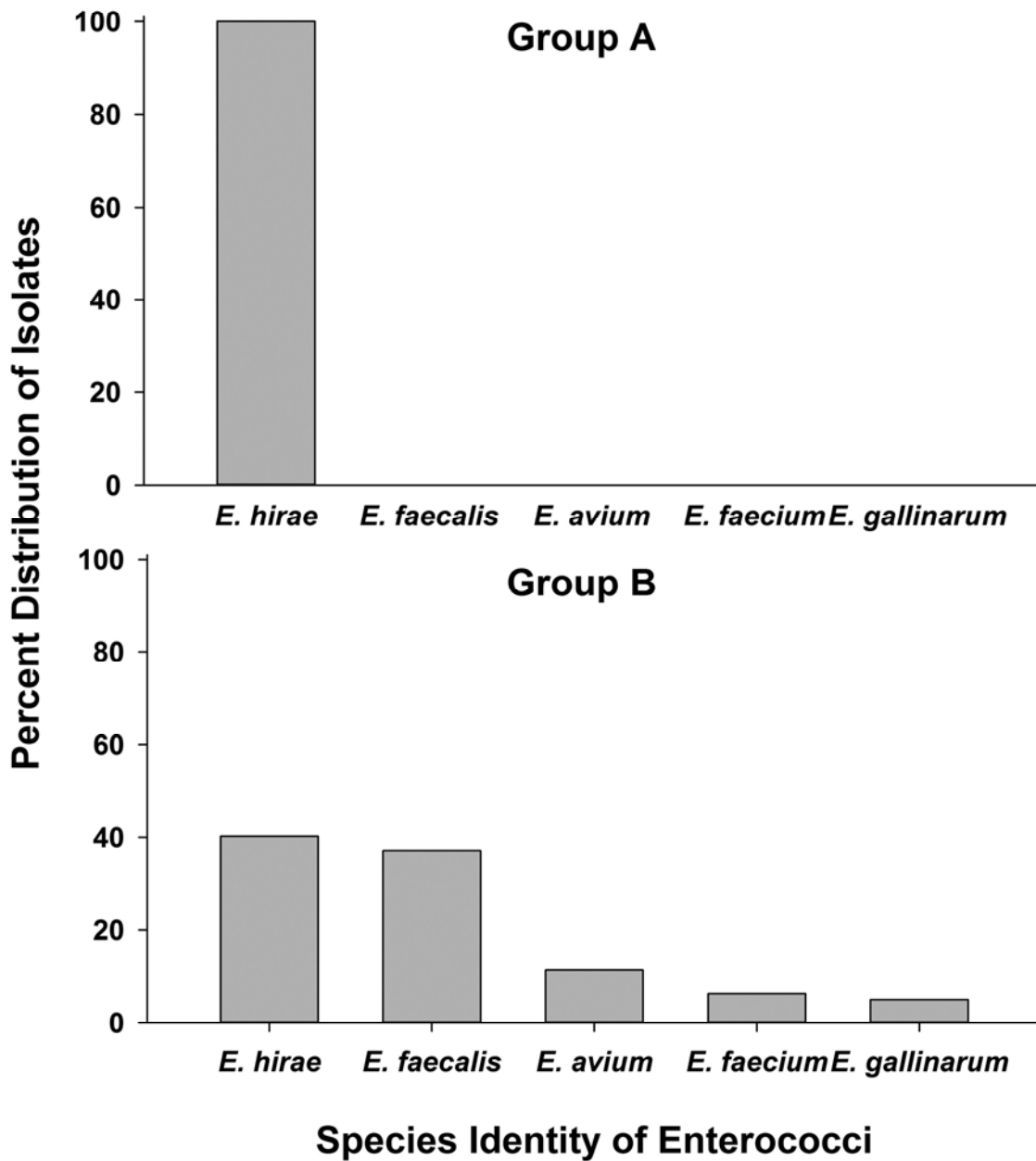
Figure 4 Population diversity of enterococcal species from 12 Group A (n=120 isolates) and 13 Group B (n=159 isolates) kittens each of which had ≥ 8 individual isolates identified as to species. Four common species, *E. faecalis, E. faecium, E. casseliflavus,* and *E. gallinarum* were identified using multiplex PCR (33). PCR amplification and sequencing of the *sodA* gene was carried out for isolates not identified by multiplex PCR (38).
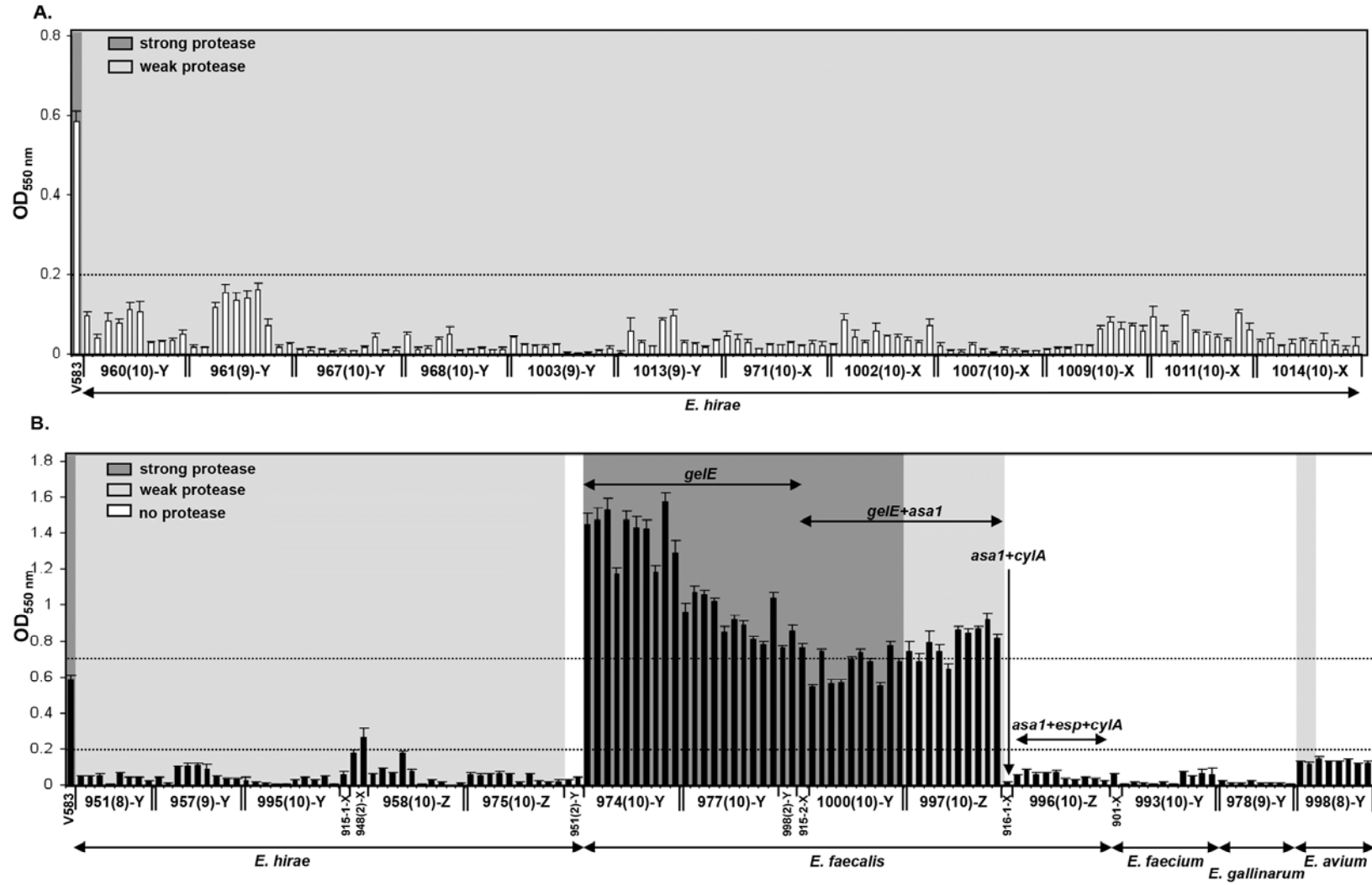
Figure 5

Figure 5   Correlation among biofilm formation, gelatinase phenotype, and presence of virulence genes (*gelE*, *asa1*, *esp*, and *cylA*) in enterococci isolated from 12 Group A kittens (n=120 isolates; panel A) and 13 Group B kittens (n=133 isolates). The dotted lines indicate biofilm formation activity (< 0.2 = no biofilm, 0.2-0.7 = biofilm, > 0.7 = strong biofilm). Kitten numbers are presented on the X-axis followed by the total number of characterized isolates in parentheses. Letters following the kitten numbers indicate kittens with: adherent enterococci (X); no adherent bacteria (Y); or adherent *E. coli* (Z). *E. faecalis* V583 was used as positive control. Bars correspond to the mean ± SEM of 5 replicates.
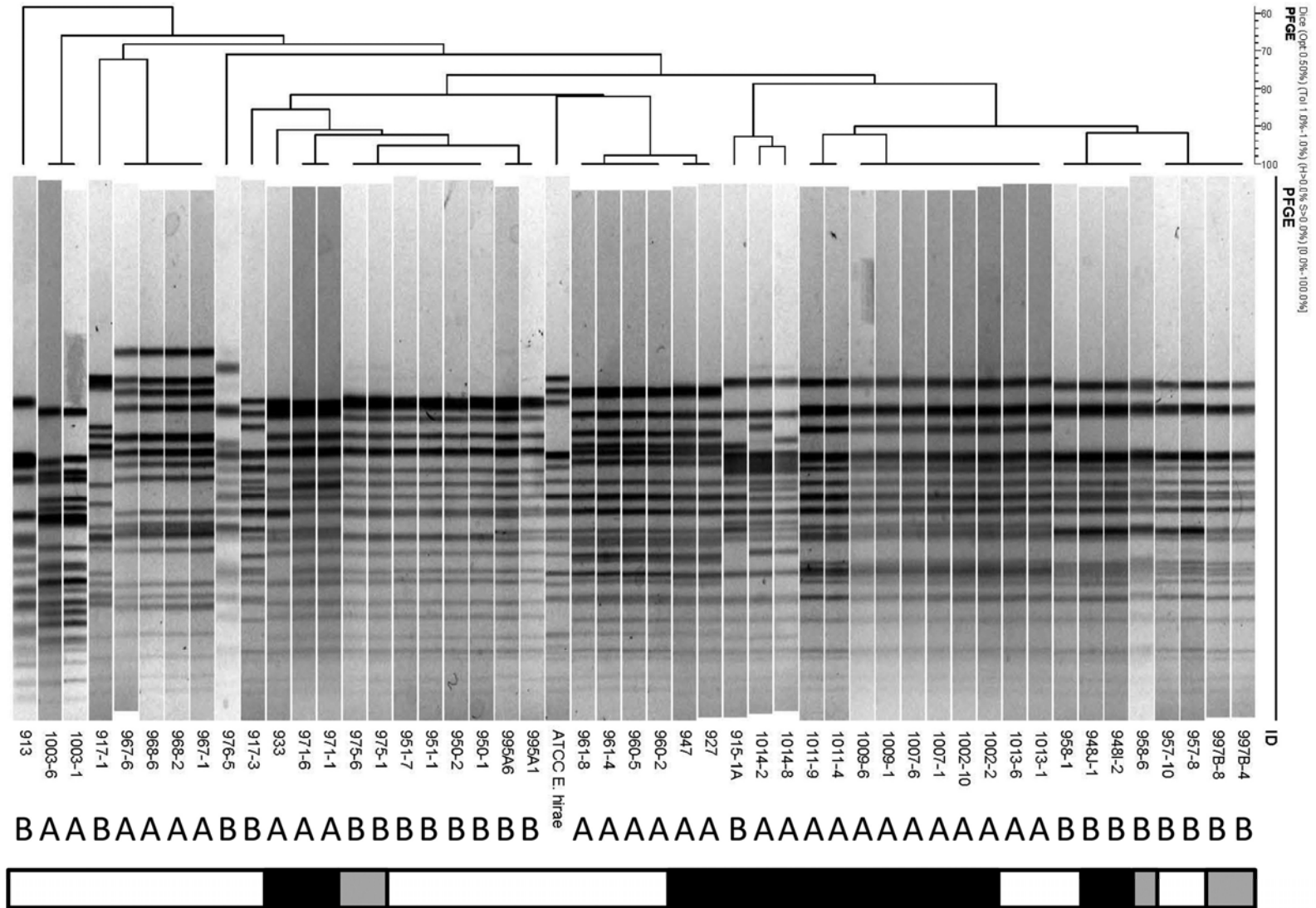
Figure 6

Figure 6　　　Pulsed-field gel electrophoresis of 48 *E. hirae* isolates cultured from the ileum mucosa of apparently healthy kittens (Group A, n=15) and kittens that died or were euthanized due to severe illness (Group B, n=12). Bar denotes light microscopic findings in kitten from which the isolate was obtained: white = no enteroadherent enterococci or EPEC observed; black = enteroadherent enterococci present; gray = EPEC present. Numerical values indicate identity of the kitten and isolate. Type strain is *E. hirae* ATCC 8043
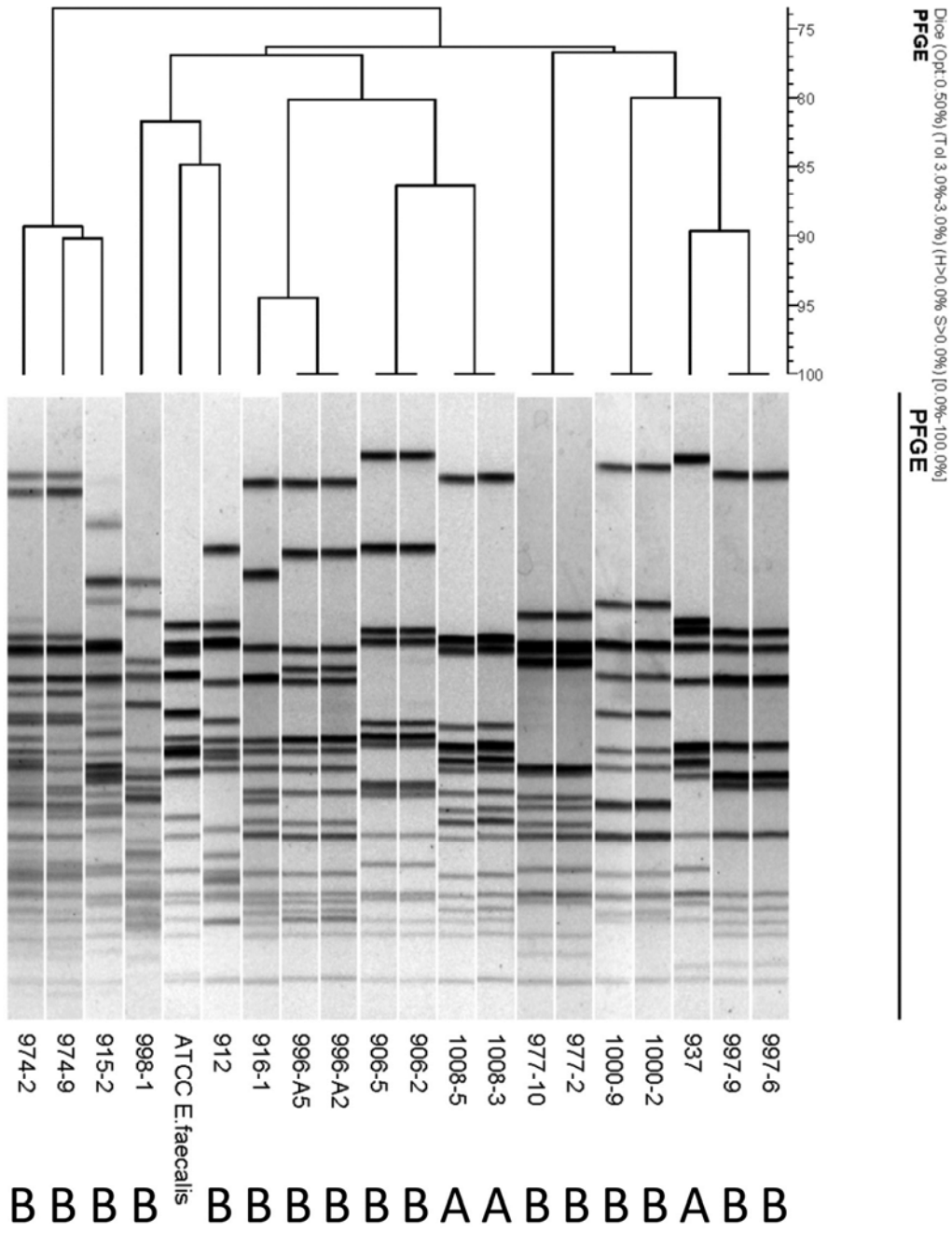
Figure 7   Pulsed-field gel electrophoresis of 19 *E. faecalis* isolates cultured from the ileum mucosa of apparently healthy kittens (Group A, n=2) and kittens that died or were euthanized due to severe illness (Group B, n=10). Numerical values indicate identity of the kitten and isolate. Type strain is *E. faecalis* ATCC 29212.
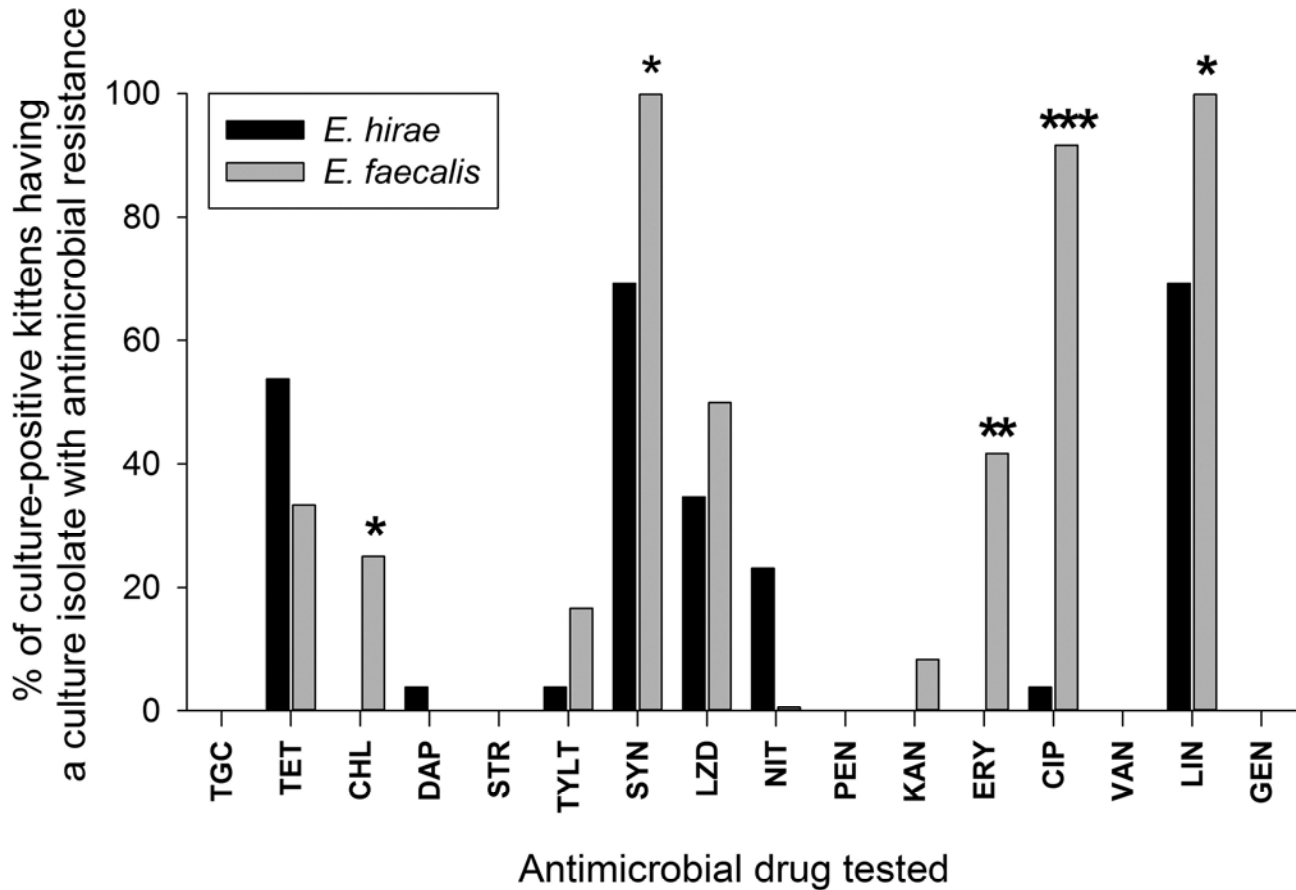
Figure 8　　　Antimicrobial susceptibility test results for ileal mucosa culture isolates of *E. hirae* (n=27) from 15 Group A and 11 Group B kittens and *E. faecalis* (n=18) from 2 Group A and 10 Group B kittens. *P <0.05, **P < 0.01, ***P< 0.001 Fischer's exact test. Tigecycline (TGC), tetracycline (TET), chloramphenicol (CHL), daptomycin (DAP), streptomycin (STR), tylosin tartrate (TYLT), quinupristin/dalfopristin (SYN), linezolid (LZD), nitrofurantoin (NIT), penicillin (PEN), kanamycin (KAN), erythromycin (ERY), ciprofloxacin (CIP), vancomycin (VAN), lincomycin (LIN), gentamicin (GEN).

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