

Winn Grant Number (W-, MT-)	W14_1616, W14-035
Project Title	Enteropathogenic <i>E. coli</i> infection in kittens – evidence for a pathogenic role in diarrheal death and protective effect of probiotics.
Institution	North Carolina State University
Investigator(s)	Jody L. Gookin, Victoria Watson

The long-term goal of our research is to identify life-saving prevention or treatment strategies to ameliorate the death toll of gastrointestinal illness in foster-age kittens. Approximately ~82 million owned and 70 million feral cats currently reside in the United States. Population projections estimate that these cats give birth to roughly 180 million kittens per year. Each year inestimable numbers of these kittens are abandoned, orphaned, or relinquished shortly after birth to be fostered by 4,000 to 6,000 U.S. animal shelters. While the exact statistics are unknown, ~15% of kittens fostered by these shelters will die or be euthanized because of illness before they reach 8-weeks of age. An obvious cause of death is unknown in as many as 50% of kittens, however many are reported to have diarrhea at the time of death or post-mortem evidence of enteritis.

Our approach has focused on identifying infectious causes of diarrheal death in kittens; to develop diagnostic tests that facilitate detection of these agents, and to establish effective treatments for their cure or prevention. We initially focused our attention on “adherent bacteria” as probable pathogens based on a series of cases in which bacteria were observed to intimately associate with the intestinal epithelium in kittens dying with diarrhea. In our first Winn study (W09-022) we identified that two different types of bacteria are associated with adherence to the intestinal epithelium of kittens – the first, *Enterococcus hirae*, is found to adhere more robustly in healthy kittens (Figure 1). The second, *E. coli*, is found to adhere only in kittens that have died or were euthanized due to a terminal illness(Figure 2)¹. What we found particularly interesting was that *E. hirae* was never observed in sick kittens with adherent *E. coli*. This suggested to us that *E. hirae* might be capable of “outcompeting” *E. coli* for colonization of the intestinal epithelium and thereby serve as an endogenous “probiotic” that protects kittens from the pathogenic effects of *E. coli* infection.

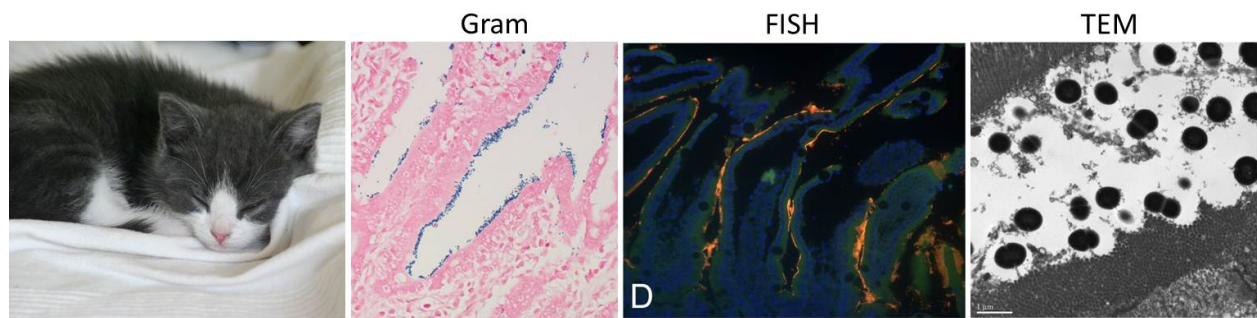


Fig 1 *Enterococcus hirae* colonizes the small intestinal epithelium of healthy kittens. In healthy kittens \leq 12-wks of age, Gram positive cocci are observed to extensively colonize the small intestinal epithelium, are identified by fluorescence in-situ hybridization (FISH) as enterococci (using a species-specific probe) and intimately associate with epithelial cells as demonstrated by transmission electron microscopy (TEM). From Nicklas et al¹.

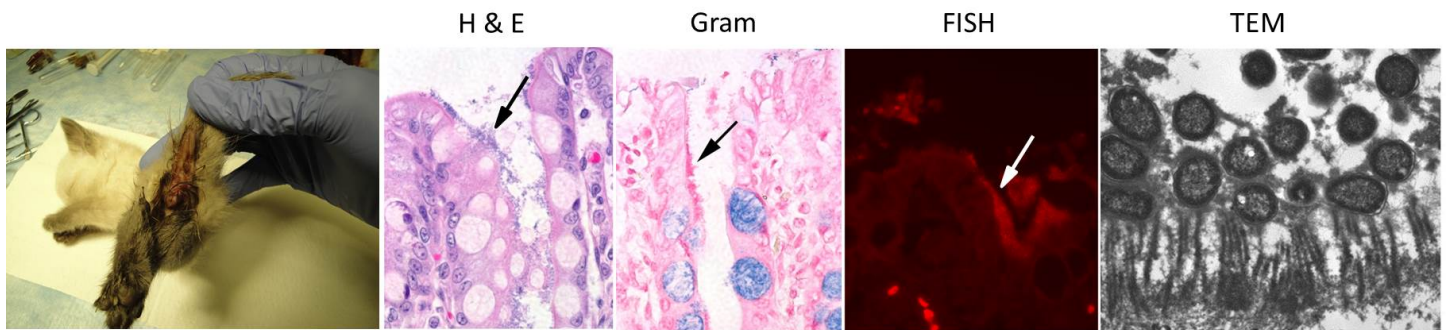


Fig 2 Enteroadherent *E. coli* is associated with mortality in foster kittens. In terminally ill kittens \leq 12-wks of age, Gram negative rods are observed to extensively colonize the small intestinal epithelium, are identified by fluorescence in-situ hybridization (FISH) as *E. coli* and intimately associate with epithelial cells as demonstrated by electron microscopy (TEM). From Nicklas et al¹.

The Gram-positive enterococci are considered to be gastrointestinal commensals and are frequently administered to kittens as probiotics. Having recognized that adherence of *E. hirae* to the intestinal epithelium was observed to be greatest in healthy kittens, we sought to further our understanding of the impact of enterococci in health and disease in this population. Therefore, our second Winn study (**W11-013**) fully characterized the mucosa-associated enterococcal flora of the small intestine of healthy and sick foster-age kittens. These studies identified *E. hirae* as the normal enterococcal flora of the small intestine in healthy kittens. In contrast, in sick kittens *E. hirae* was commonly displaced by *Enterococcus faecalis* (Figure 3)².

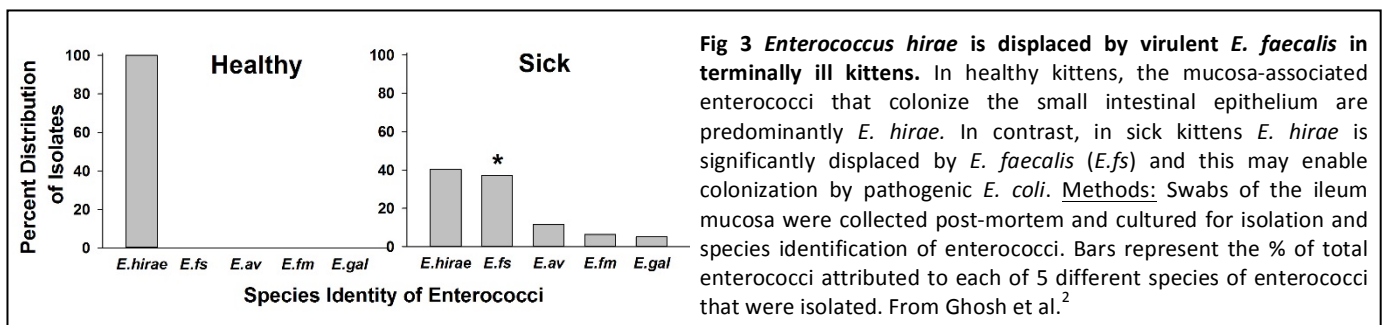


Fig 3 *Enterococcus hirae* is displaced by virulent *E. faecalis* in terminally ill kittens. In healthy kittens, the mucosa-associated enterococci that colonize the small intestinal epithelium are predominantly *E. hirae*. In contrast, in sick kittens *E. hirae* is significantly displaced by *E. faecalis* (*E.fs*) and this may enable colonization by pathogenic *E. coli*. Methods: Swabs of the ileum mucosa were collected post-mortem and cultured for isolation and species identification of enterococci. Bars represent the % of total enterococci attributed to each of 5 different species of enterococci that were isolated. From Ghosh et al.²

The *E. faecalis* bacteria that we isolated from sick kittens were strong biofilm-formers, carried multiple genetic virulence determinants, and were multiple-drug resistant. These studies suggest that dysbiosis of the mucosa-associated enterococci may enable colonization by pathogenic *E. coli*. In addition, *E. faecalis* may be a direct or indirect contributor to mortality in these kittens.

Based on our observation that adherence of *E. coli* was exclusively and significantly associated with mortality in these kittens, we obtained our Winn study (**W11-012**) to determine the specific *E. coli* pathotypes that are infecting these kittens and the role of concurrent gastrointestinal infections as contributing causes to their mortality. We targeted the inclusion of kittens with clinical signs of diarrhea and apparently-healthy kittens while under foster care. We additionally collected fecal samples from live kittens with and without diarrhea for inclusion in the study. For each of these kittens, feces were cultured for isolation of *E. coli* and isolates were screened by PCR to “survey” these bacteria for the presence of key virulence factors that would categorize them into major pathotypes of diarrhea-associated *E. coli*. Our results demonstrate a high prevalence of diarrhea-causing *E. coli* in the feces of both healthy and sick kittens. Isolation of Enteropathogenic *E. coli* (EPEC) was of particular interest because these *E. coli* are capable of adherence to the intestinal epithelium and we have

shown that adherence of *E. coli* is associated with mortality in foster-age kittens. Thus far these pathogenic isolates are being found via fecal culture in both the healthy and sick kitten populations. What we don't know is whether the mere presence of EPEC, the numbers of EPEC present, or the complement of virulence factors carried by EPEC are the most important determinants of their pathogenicity. Therefore we obtained our current Winn Feline Foundation grant (**W14_1616**) to help answer some of these questions. Currently there is no way to diagnose or quantify *EPEC* infection without performing fecal bacterial culture, randomly selecting *E. coli* colonies and screening each one for pathogenicity determinants. This is a major expense and time impediment to our ability to diagnose or better understand the clinical significance of *EPEC* infections in kittens. With our grant support we have established that a simple quantitative PCR test, performed on kitten feces, can be used to detect the presence of genes that are unique to *E. coli* with adherent abilities. Adherence of *EPEC* to the intestinal epithelium appears to be a key mechanism of pathogenicity and therefore the presence of this gene in feces indicates the presence of *E. coli* with a potential to cause diarrhea. We have demonstrated that this gene is found more commonly and in higher amounts in the feces of kittens with diarrhea than those without diarrhea and appears to have a significant potential to serve as a "biomarker" of risk for diarrhea-associated mortality in kittens. We have yet to identify any indirect way (via fecal examination or characterization of *EPEC* isolates) to identify if *EPEC*, when present in feces, is the direct cause of diarrhea. Because *EPEC* can also be found in kittens without diarrhea and in kittens with other causes of diarrhea, we are currently looking for unique characteristics of *EPEC* that will enable us to judge their significance when detected in an individual kitten. Based on characterization of kitten *EPEC* isolates so far, it appears that the bacteria are genotypically and phenotypically diverse. Therefore, determining the significance of *EPEC* still remains a challenge and a puzzle we continue to work to solve.

1. Nicklas JL, Moisan P, Stone MR, et al. In situ molecular diagnosis and histopathological characterization of enteroadherent *Enterococcus hirae* infection in pre-weaning-age kittens. *J Clin Microbiol* 2010;48:2814-20.
2. Ghosh A, Borst L, Stauffer SH, et al. Mortality in kittens is associated with a shift in ileum mucosa-associated enterococci from *Enterococcus hirae* to biofilm-forming *Enterococcus faecalis* and adherent *Escherichia coli*. *J Clin Microbiol* 2013;51:3567-78.