ABSTRACT

WATSON, VICTORIA ELIZABETH. Prevalence, Genotypic and Phenotypic Characteristics, and Pathogenic Effects of Enteropathogenic E. coli in Naturally and Experimentally Infected Kittens (under the direction of Dr. Jody Gookin)

In children and kittens, infectious diarrhea is a leading cause of death. In children, enteropathogenic E. coli (EPEC) is a common cause of diarrhea and is associated with increased risk of mortality. The prevalence of typical EPEC is decreasing whereas atypical EPEC (aEPEC) is increasing and contemplated to be an emerging pathogen in children. However, aEPEC can be isolated from children with and without diarrhea and mechanisms responsible for diarrhea are unclear. In an autopsy-based study of kitten mortality in U.S. animal shelters, we identified an association between death and colonization of intestinal epithelium by EPEC. We hypothesized that EPEC is significantly associated with diarrheal mortality in kittens and studies to characterize infection in kittens would provide insights with translational significance to children. Accordingly, we aimed to 1) determine the prevalence and type of EPEC infection in kittens and identify factors associated with disease, 2) determine the genomic similarity of kitten EPEC to human EPEC and identify phenotypic or genotypic characteristics of kitten EPEC isolates that can distinguish pathogenic from non-pathogenic strains, and 3) determine pathogenic effects of EPEC following experimental infection of kittens and the influence of the microbiome and a commensal member of the microbiome, Enterococcus hirae, on clinical manifestations of infection.

We performed a prospective, case-controlled study of EPEC infection in shelter kittens with and without diarrhea. EPEC was diagnosed by fecal culture of EPEC and qPCR amplification of intimin (eae) from fecal DNA. Gastrointestinal pathology and medical records were evaluated for associations with EPEC. EPEC were serotyped, characterized using pulsed-
field gel electrophoresis (PFGE), and sequenced for genomic comparison to human EPEC. Phenotypic characteristics of EPEC were determined by quantifying biofilm formation and bacterial motility and identifying adherence to HEp-2 cells. Kittens were experimentally infected with aEPEC under conditions of unaltered intestinal microbiota (n=8) or antibiotic-altered microbiota (n=8). Kittens were randomized to receive either E. hirae or vehicle prior to or concurrent with infection. The effect of EPEC on intestinal function was assayed by measuring fecal consistency and quantifying intestinal absorption and permeability.

The EPEC isolated from shelter kittens was confirmed to be aEPEC and comparably to children, kittens with and without diarrhea shed aEPEC. Based on culture results, overall prevalence of aEPEC in kittens was 17%. Using qPCR to estimate colony forming units of aEPEC, we demonstrated a greater quantity of aEPEC in kittens with diarrhea. Furthermore, the presence of eae was significantly associated with more severe microscopic lesions in the small intestine and colon, characterized by increased inflammation and increased villous epithelial cell loss. Kittens with aEPEC infection were significantly more likely to have received parenteral fluid administration, indicating an association between aEPEC infection and severity of dehydration. Kitten aEPEC were genetically diverse and phylogenomically indistinguishable from human aEPEC isolates. Genotypic or phenotypic characteristics did not distinguish pathogenic from non-pathogenic aEPEC. Experimental infection in kittens without antibiotic-altered microbiota did not produce clinical signs but kittens had significantly altered intestinal function, demonstrated by decreased absorption and permeability. Experimental infection in kittens with antibiotic-altered microbiota caused increased fecal consistency. The probiotic composed of E. hirae exerted no adverse effects on kittens nor altered response to aEPEC infection.
These studies identify that aEPEC is prevalent in kittens and quantity of aEPEC is associated with diarrhea and increased intestinal inflammation. Kitten aEPEC are phenotypically and genotypically diverse and phylogenomically indistinguishable from human aEPEC isolates. Accordingly, kittens may serve as a reservoir for potentially zoonotic aEPEC. Although experimental infection caused limited signs, findings indicate a role for the microbiome in susceptibility to aEPEC-associated diarrhea. Naturally-occurring aEPEC infection in kittens may represent a novel animal model of aEPEC infection in children.
Prevalence, Genotypic and Phenotypic Characteristics, and Pathogenic Effects of Enteropathogenic \textit{E. coli} in Naturally and Experimentally Infected Kittens

by

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BIOGRAPHY

Victoria Elizabeth Watson was born on June 2nd 1981 in Baltimore, Maryland. She was quickly given the more appropriate and shorter nickname of Tory. She lived in a small town in Maryland called Walkersville until she turned 12. At that time, her father moved the family, much to her 16-year-old sister’s chagrin, to Charleston, South Carolina. Tory attended Ashley Hall School and spent numerous afternoons at her mother’s desk in a research laboratory at the Medical University of South Carolina. Although she was immersed in research science (even dinner conversations), she was focused on becoming a veterinarian. She graduated from the University of South Carolina in 2003 and after a short hiatus working as a veterinary assistant in a small animal hospital, she was accepted as a member of the Class of 2009 at the University of Georgia, College of Veterinary Medicine (CVM). During vet school, she became very interested in anatomic pathology and was commonly found on the necropsy floor or around a multi-headed scope in the pathology department. Thus, following completion of a small animal rotating internship at Hickory Veterinary Hospital in Plymouth Meeting PA, she returned to UGA for her residency in Anatomic Pathology. She was board certified by the American College of Veterinary Pathologists in 2014. Tory’s interest in research has evolved over the years but she has always been interested in translational models of infectious disease. She started as a graduate student at North Carolina State University in Comparative Biomedical Sciences at the CVM. Her research was under the direction of Dr. Jody Gookin and has been focused on defining the role of enteropathogenic *E. coli* in diarrhea and mortality in kittens and investigating the possible protective benefit of a commensal organism in the gastrointestinal tract, *Enterococcus hirae*. 
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TABLE OF CONTENTS

List of Tables ........................................................................................................................................vii

List of Figures .........................................................................................................................................ix

Chapter I: The pathogenesis of Enteropathogenic *Escherichia coli* ..............................................1

Introduction.............................................................................................................................................1

EPEC in children ...................................................................................................................................4

Typical versus atypical EPEC .............................................................................................................7

Adherence of EPEC .............................................................................................................................12

EPEC alters intestinal permeability and absorption ......................................................................15

EPEC effectors associated with cell death .........................................................................................18

EPEC effectors that modulate the innate immune response ..............................................................19

Host defense against EPEC ...............................................................................................................24

Experimental and naturally-occurring animal models of EPEC ......................................................29

Treatment and Prevention of EPEC .................................................................................................41

Tables ..................................................................................................................................................44

Chapter II: Association of atypical enteropathogenic *Escherichia coli* with diarrhea-related mortality in kittens .................................................................46

Abstract...............................................................................................................................................47

Introduction...........................................................................................................................................48

Materials and methods ......................................................................................................................51

Results................................................................................................................................................58

Discussion............................................................................................................................................64
References
# LIST OF TABLES

## CHAPTER I

**Table 1.** Virulence effectors of EPEC that influence adhesion to epithelial cells

**Table 2.** Virulence effectors of EPEC that modulate the innate immune response

## CHAPTER II

**Table 1.** Demographic data from 61 live kittens with and without clinical signs of diarrhea

**Table 2.** Demographic data from 54 deceased kittens that were apparently healthy or died or were euthanized due to diarrhea

**Table 3.** Results of multiplex PCR for detection of virulence gene(s) in E. coli cultured from feces of apparently healthy kittens and kittens that died or were euthanized due to diarrhea

**Table 4.** Gross and light microscopic lesion scores obtained from apparently healthy kittens and kittens that were euthanized or died due to severe diarrhea and on the basis of EPEC culture and/or qPCR for eae

**Table 5.** Results of parasitological examination(s) of gastrointestinal samples obtained at the time of autopsy from 51 kittens

**Table 6.** Comparison of medical records data from live and deceased kittens for factors associated with positive results of EPEC culture and/or qPCR for eae

**Supplementary Table 1.** Most common gross lesions observed at autopsy in 39 kittens and corresponding light microscopic lesions observed in the same region of intestine
**Supplementary Table 2.** Detailed list of medications and treatments administered to kittens in the study………………………………………………………………………………………………………81

**CHAPTER III**

**Table 1.** Characterizations of kitten EPEC…………………………………………………………………………………………………………………………110

**Table 2.** Distribution of serotypes among 24 EPEC isolates from different clinical outcomes examined in the study………………………………………………………………………………………………………111

**Table 3.** Number of gene clusters identified using LS-BSR in relation to species of origin (cat versus human) and clinical outcome………………………………………………………………………………………………………………………………………………112

**Table 4.** Presence of locus of enterocyte effacement (LEE) genes, on the basis of PCR amplification, in 12 kitten EPEC isolates…………………………………………………………………………………………………………………………………………………………113

**CHAPTER IV**

**Table 1.** Comparison of cultured EPEC from kittens that received or did not receive probiotics…………………………………………………………………………………………………………………………………………………………145
LIST OF FIGURES

CHAPTER II

Figure 1. Quantitative PCR (qPCR) amplification of enterocyte attaching and effacing (eae) gene from fecal DNA of 61 live kittens with and without diarrhea........................................82

Figure 2. Quantitative PCR (qPCR) amplification of enterocyte attaching and effacing (eae) gene from fecal DNA of apparently healthy kittens euthanized by animal control and kittens that died or were euthanized because of severe diarrhea while in foster care......................83

Figure 3. Small intestinal and colonic histopathology lesion scores based on results of fecal culture and qPCR for eae in apparently healthy kittens and those dying or euthanized due to severe diarrhea.................................................................................................................84

Figure 4. Representative photomicrographs of lesions significantly associated with presence of EPEC (culture and/or eae qPCR) in kittens..............................................................85

Figure 5. Representative fluorescence microscopy images of focally adherent E. coli in the small intestine of one kitten (A – D) and the colon of another kitten (E – H) that were euthanized due to severe diarrhea. ........................................................................................................86

Supplementary Figure 1. Representative photographs of common gross lesions identified in kittens that died or were euthanized due to diarrhea.................................................................87

Supplementary Figure 2. Representative photograph and photomicrographs of commonly identified parasites. ...........................................................................................................................88

CHAPTER III

Figure 1. Pulsed-field gel electrophoresis (PFGE) of 16 kitten EPEC isolates obtained from cases of asymptomatic (AI) and lethal (LI) infection. .................................................................114
Figure 2. Kitten EPEC isolates contain plasmids of varying sizes.........................115

Figure 3. Phylogenomic analysis of 12 kitten EPEC isolates associated with asymptomatic or lethal infections compared with previously sequenced EPEC genomes and diverse reference genomes of E. coli and Shigella isolates.................................................................116

Figure 4. Multiple virulence genes associated with pathogenicity of EPEC and/or EHEC are variably present in EPEC from kittens.................................................................117

Figure 5. Kitten EPEC isolates form biofilm.............................................................118

Figure 6. Motility assay shows a wide range of motility of kitten EPEC.....................119

Figure 7. Fluorescence actin staining assay demonstrates epithelial cell adherence of kitten EPEC isolates.................................................................120

CHAPTER IV

Figure 1. Experimental timeline for studies.............................................................146

Figure 2. Confirmation of aEPEC infection in kittens.................................................147

Figure 3. Designation, serotype, and pulse-field gel electrophoresis pulsotype of the 3 isolates used for experimental infection and 2 isolates carried by kittens at the time of purchase by a commercial vendor.................................................................148

Figure 4. Kittens that did not receive antibiotics prior to infection remained clinically healthy throughout the course of the experiment.................................................................149

Figure 5. Intestinal function assays to determine the impact of EPEC infection or probiotic administration to kittens with no antibiotic-induced alteration of the microbiota...........151
**Figure 6.** Kittens that received antibiotics prior to infection remained clinically healthy but showed significant increases in their fecal consistency during antibiotic administration and 3 days post-infection..............................................................152

**Figure 7.** Intestinal function assays to determine the impact of EPEC infection in kittens with antibiotic-induced alteration of the microbiota............................................................154

**Figure 8.** Effect of the probiotic composed of *E. hirae* on the shedding of *E. hirae* in kittens that did not receive antibiotics prior to probiotic administration (first study)......................155
CHAPTER 1

The pathogenesis of Enteropathogenic Escherichia coli

Introduction

Diarrhea is one of the leading causes of death in children under 5 years of age. Each year, diarrhea claims approximately 900,000 lives worldwide, with most fatalities occurring in developing countries (Black et al., 2010; Kirk et al., 2015; Kotloff et al., 2013; Liu et al., 2012). In developing countries, where infectious causes of diarrhea are highest, leading etiologies of diarrhea are the diarrheagenic Escherichia coli (DEC) (Nataro and Kaper, 1998; Nataro and Martinez, 1998). The group of DEC includes enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), enterotoxigenic E. coli (ETEC), enteroaggregative E. coli (EAEC), enteroinvasive E. coli (EIEC), and diffusely adhering E. coli (DAEC). Although the use of oral rehydration therapy has helped to decrease fatalities due to diarrhea, EPEC, EHEC, and ETEC remain important causes of diarrhea and death in children (Ochoa and Contreras, 2011).

In children, EPEC has been connected to 17 million cases of diarrhea in a single year (Kirk et al., 2015). EPEC was first discovered as an agent of diarrhea in children in the 1940’s (Robins-Browne et al., 1980). The term EPEC was adopted in the 1950’s to identify the first few serotypes associated with neonatal diarrhea (Baldwin, 1998; Neter and Shumway, 1950). EPEC is one of the most common causes of persistent watery diarrhea in children; EPEC is present in up to 63% of children with persistent diarrhea (Abba et al., 2009). Diarrhea leads to malnutrition and dehydration and in children that survive this onslaught of diarrhea, can even lead to lasting mental and health issues (Kirk et al., 2015;
Lorntz et al., 2006). EPEC causes disease via mechanisms unique to the bacteria. In humans, EPEC predominantly colonizes the small intestine. Once it reaches the small intestine, it lightly adheres to the epithelial cells lining the villi. Then EPEC produces a type III secretion system, composed of multiple *E. coli* secreted proteins (Esp’s), which inserts an adherence receptor (translocated intimin receptor) to bind to the adhesin, intimin (Kenny et al., 1997). This produces a tight adherence which is one of the main pathogenic characteristics of EPEC, termed the attaching and effacing (A/E) lesion (Knutton et al., 1989; Rothbaum et al., 1982; Ulshen and Rollo, 1980). This A/E lesion is characterized by adherence of the bacteria to the enterocyte and microvillus damage leading to loss of absorptive surface along the apical surface of the enterocyte and impaired nutrient absorption (Jerse et al., 1990; McNeish et al., 1975). The A/E lesion also leads to actin polymerization and changes in cytoskeletal structure creating a “pedestal” subjacent to the bacteria and subsequent changes in cell shape and permeability (Knutton et al., 1989). Additional virulence factors have been identified that lead to functional changes in epithelial cells, altering permeability, absorption, and electrolyte transport.

Research regarding the pathogenesis of EPEC has focused primarily on *in vitro* cell culture models and two small animal models, mouse and rabbit. These models have been pivotal in discovering the steps of adherence, the effectors of EPEC that affect epithelial cell function that could lead to diarrhea, and mechanisms of immune defense in EPEC. Studies of EPEC pathogenesis using these models support the presence of effector proteins that can alter permeability of the tight junction barrier, electrolyte transport, apical plasma membrane microvillus architecture and function, and promote secretion of pro-inflammatory cytokines.
In addition to these models, future research is likely to advance to studies of therapies and preventative measures using other possibly naturally occurring animal models.
EPEC in children

Histologically, children with persistent diarrhea and EPEC have villus atrophy and overall decreased mucosal thickness (Fagundes-Neto et al., 1997; Rothbaum et al., 1982; Ulshen and Rollo, 1980). These lesions, specifically villus atrophy, are likely associated with loss of mucosal absorption further confounding the loss of absorptive surface associated with the A/E lesion of EPEC. EPEC also leads to loss of barrier function and increased permeability due to its effects on the epithelial cell cytoskeleton and tight junction proteins (Glotfelty and Hecht, 2012; Shifflett et al., 2005; Simonovic et al., 2000; Thanabalasuriar et al., 2010). Therefore, diarrhea is likely also occurring due to increased loss of water. It is unclear which mechanism of diarrhea is more important in the disease pathogenesis and mortality risk of EPEC. Further confounding the role of EPEC in diarrhea-associated mortality, EPEC can be found in both children with diarrhea and asymptomatic children (Acosta et al., 2016; Alikhani et al., 2006; Barletta et al., 2011; Donnenberg et al., 2015; Hazen et al., 2016). Nonetheless, EPEC remains a predominant cause of diarrhea and mortality in infants.

Another important differentiation in EPEC causing disease in children is the presence of an extra-chromosomal plasmid known as the EPEC adherence factor. This EPEC adherence factor plasmid (EAF) is present in typical EPEC, but is absent in atypical EPEC (Goffaux et al., 1997; Nataro and Martinez, 1998). The EAF plasmid contains a gene that encodes a type IV fimbria, called bundle forming pilus (bfp). The role of BFP is to form links between bacteria and thus formation of microcolonies of bacteria (Tobe, 2010; Tobe and Sasakawa, 2002). In children, typical EPEC is more predominantly studied as a cause of
diarrhea. Typical EPEC continues to be an important pathogen in developing countries; whereas, atypical EPEC is becoming a higher concern and is considered an emerging pathogen in both developing and developed countries (Kotloff et al., 2013; Sampaio et al., 2014).

The prevalence of EPEC in children varies worldwide. In general, prevalence studies culture feces for isolation of *E. coli* and perform PCR for detection of the gene that encodes intimin (*eae*) to identify the *E. coli* as EPEC. In sub-Saharan Africa EPEC is one of the top diarrheagenic agents and can be found in 11-13% of children with diarrhea analyzed for infectious causes of diarrhea (Chiyangi et al., 2017). In India, EPEC was found to be highly associated with diarrhea in all ages; EPEC was found in 42% of the samples collected from 300 people with diarrhea (Purwar et al., 2016). However, another study found EPEC in only 2% of the fecal samples examined from patients with diarrhea (Dutta et al., 2013). In Peru, EPEC prevalence in children aged 6 – 19 months, is approximately 14% (Acosta et al., 2016). In that study, EPEC was the most prevalent diarrheagenic *E. coli* in children with diarrhea (prevalence of 28%) (Acosta et al., 2016). In a per-urban suburb of Mexico City, atypical EPEC, but not typical EPEC was significantly associated with diarrhea in infants (Estrada-Garcia et al., 2009). In Norway, a developed country, atypical EPEC was not significantly associated with diarrhea as it was identified in 15% of samples from children with diarrhea and 10% of normal control samples (Afset et al., 2004). However, in that study, atypical EPEC was associated with longer duration of diarrhea (14 days or more) (Afset et al., 2004). Likewise, EPEC is not considered a significant cause of diarrhea in children in the UK (Knutton et al., 2001). The wide variability in the prevalence and association of EPEC
with diarrhea is likely due to differences in sanitation practices in developing vs. developed countries. Additionally, the prevalence of EPEC has been demonstrated to be influenced by the age of the child, with increased prevalence in children under 2 years of age, but also increased prevalence in infants over the age of 6 months (Ochoa et al., 2011).
Typical versus atypical EPEC

Strains of EPEC are further defined as typical or atypical based on the presence or absence of the *E. coli*/EPEC adherence factor plasmid (EAF) with the gene (*bfp*) encoding the type IV bundle forming pilus (Kaper et al., 1997). In addition to *bfp*, the plasmid also contains *perA*, *perB*, and *perC* which are associated with regulation of *bfp* expression as well as enhanced transcription of genes on the LEE, e.g. *eae* (Gomez-Duarte and Kaper, 1995). Detection of *bfp* or EAF by PCR is performed to determine the type of EPEC. EPEC strain type is also determined by the type of adherence displayed by the bacteria in co-culture with epithelial cell lines (e.g. HeLa, HEp-2) (Cravioto et al., 1991; Nataro and Kaper, 1998; Scaletsky et al., 2002; Shariff et al., 1993). Typical EPEC strains adhere almost exclusively in a localized-adherence (LA) pattern, whereas atypical EPEC strains adhere in LA-like (LAL), diffuse adherence (DA), or aggregative adherence (AA) patterns (Pelayo et al., 1999; Trabulsi et al., 2002). The difference in adhesion is directly linked to the presence of the plasmid (Bueris et al., 2015; Jerse et al., 1990). Lastly, some researchers have used serotyping to differentiate EPEC strains. However, many serotypes, even those considered “classical,” can be either atypical or typical (Trabulsi et al., 2002; Vieira et al., 2001). Furthermore, serotyping is not considered sufficient to diagnose EPEC or other diarrheagenic *E. coli* as it does not determine virulence.

Typical EPEC is well-established as an enteropathogen (Nataro and Kaper, 1998). Typical EPEC are common causes of diarrhea in children < 5 years in developing countries (Scaletsky et al., 2002). The Global Enteric Multicenter Study (GEMS) determined that tEPEC is significantly associated with mortality (hazard ratio of 2.6) in infants (Kotloff et al.,
Another study similarly showed that EPEC (not separated into typical and atypical) was associated with higher risk of mortality in children (Pires et al., 2015). In human experimental infection (adults), the typical EPEC strain E2348/69 (at a dose of $10^{10}$) caused diarrhea in 3/5 adults while the strain E851/71 caused diarrhea in all adults at all doses (Levine et al., 1978). Out of 10 human adults orally infected with a strain of E2348/69 lacking the EAF plasmid, only 2 had diarrhea, as compared to 9/10 adults infected with E2348/69 with retained plasmid (Levine et al., 1985). However, studies over the past 2 decades are showing a decline in cases of typical EPEC and an increase in atypical EPEC (Araujo et al., 2007; Bakhshi et al., 2013; Bueris et al., 2007; Canizalez-Roman et al., 2016; Contreras et al., 2012; Hu and Torres, 2015; Liebchen et al., 2011; Moreno et al., 2010; Nair et al., 2010; Ochoa et al., 2009; Trabulsi et al., 2002; Vilchez et al., 2009; Wani et al., 2006).

Atypical EPEC, in comparison to typical EPEC, is not as well-established as a pathogen and its role in diarrhea is controversial. Atypical EPEC is present in both developed and developing countries (Santona et al., 2013) and has been identified in multiple species with and without clinical signs of infection (Hernandes et al., 2009; Trabulsi et al., 2002). In the United States, atypical EPEC was the only type of EPEC identified in stool from children and was present in both children with diarrhea and asymptomatic controls (Vernacchio et al., 2006). A similar prevalence in children with and without diarrhea has likewise been shown in other countries (Aslani and Alikhani, 2011; Bueris et al., 2007; Contreras et al., 2012; Ghosh and Ali, 2010; Nguyen et al., 2005; Ochoa et al., 2009; Rodrigues et al., 2004b; Vilchez et al., 2009). However, in contrast, studies have shown that atypical EPEC is significantly associated with diarrhea (Afset et al., 2003; Afset et al., 2004; Estrada-Garcia et al., 2009;
Moreno et al., 2010; Robins-Browne et al., 2004). In one study in Brazil on the prevalence of *E. coli* pathotypes in children < 5 years-old presenting with diarrhea, atypical EPEC was the second most prevalent etiology and was significantly associated with diarrhea (Araujo et al., 2007). In Iran, both typical and atypical EPEC were determined to be significantly associated with diarrhea (Alikhani et al., 2006). In Norway, atypical EPEC has been associated with prolonged (> 14 days) diarrhea in children (Afset et al., 2004; Nguyen et al., 2006). A study in Germany, which included only children with diarrhea, identified atypical EPEC in 11.3% of the patients and they isolated only one typical EPEC strain (Kozub-Witkowski et al., 2008). When compared to other enteropathogens, atypical EPEC was significantly associated with diarrhea in infants in England (Sakkejha et al., 2013). An age difference in prevalence was similarly identified in children with diarrhea in India and Mexico; atypical EPEC prevalence was highest in children < 2 years of age (Canizalez-Roman et al., 2016; Malvi et al., 2015). In children < 2 years of age, atypical EPEC is actually more prevalent in those > 6 months, which may be associated with increased breast-feeding in that age group (Ochoa et al., 2009).

Another reason for the controversy of atypical EPEC as a cause of diarrhea is that the strains are very heterogeneous (Afset et al., 2008; Scaletsky et al., 2009; Tennant et al., 2009; Vieira et al., 2001). Therefore, studies have recently started reporting genetic backgrounds of atypical EPEC which are significantly associated with diarrhea. It is becoming clear that atypical EPEC, although containing the LEE and adhering to intestinal epithelial cells similarly to typical EPEC, also can acquire virulence factors from other diarrheagenic *E. coli* (Bando et al., 2009). This is particularly true for atypical EPEC within phylogenetic group
B1, the group of atypical EPEC that was demonstrated to be significantly more prevalent in people with diarrhea (Wang et al., 2013). Of the acquired virulence factors, the presence of the gene encoding the enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST-1) has been significantly associated with diarrhea in children and adults in multiple countries (Dulguer et al., 2003; Hedberg et al., 1997; Yatsuyanagi et al., 2003). In a Norwegian study, a total of 12 genes were found to be significantly associated with diarrhea and atypical EPEC (Afset et al., 2006). These included the genes encoding EAST-1, EHEC factor for adherence/lymphocyte inhibitory factor (*efa1/lifA*), EHEC hemolysin, and porcine attaching-and-effacing associated protein. Additional studies have implicated that the gene *efa1/lifA* is important for virulence of atypical EPEC (Badea et al., 2003; Narimatsu et al., 2010; Slinger et al., 2017; Vieira et al., 2010). Detection of *efa1/lifA* in fecal DNA was significantly associated with higher bacterial load (quantity of *eae*) in children with diarrhea (Slinger et al., 2017). In contrast, one study did not find a significant association between presence of *efa1/lifA* and diarrhea (Tennant et al., 2009). Detection of the genes encoding the porcine attaching-and-effacing associated protein (*paa*) and EHEC hemolysin (*ehxA*) in atypical EPEC in another study was also determined to be significantly associated with diarrhea (Scaletsky et al., 2009). Additionally the gene encoding the *E. coli* common pilus (*ecpA*) has been detected in a high number of atypical EPEC from cases of diarrhea (Vieira et al., 2016) and is associated with adherence (Hernandes et al., 2011). An additional virulence factor that has been associated with intestinal colonization is the serine protease, Pic, which was originally identified in enteroaggregative *E. coli* (Abreu et al., 2016). There are likely additional genes or characteristics influencing virulence properties of atypical EPEC. Moreover, the level of
transcription of virulence genes on the locus of enterocyte effacement can differ between strains of atypical EPEC, thus impacting adherence and pathogenesis (Rocha et al., 2011). Full genomic sequencing of strains of atypical EPEC have demonstrated specific clones and future work will further elucidate pathogenesis of these atypical EPEC (Ingle et al., 2016). Overall, the pathogenicity of atypical EPEC requires additional studies, but currently atypical EPEC appears to be an emerging pathogen, particularly in children, and certain characteristics in the bacteria itself, host, or environment are required to produce clinical signs of diarrhea.
Adherence of EPEC

Once EPEC reaches the small intestine, it begins the process of adhering to an epithelial cell along the villi. Adherence of EPEC to the host epithelial cell *in vivo* or epithelial cell line *in vitro* involves the proteins intimin and translocated intimin receptor and the Type III Secretion System (T3SS) (Table 1). The genes encoding intimin (*eae*) and Tir (*tir*) are found on a 35.6 kB section of the chromosomal DNA, termed the Locus of Enterocyte Effacement (LEE) (DeVinney et al., 1999; Franzin and Sircili, 2015; Kenny et al., 1997). Tir and intimin bind to each other, which forms the adherence of the bacterium to the host epithelial cell leading to the A/E lesion (Donnenberg and Kaper, 1991; Donnenberg et al., 1997; Donnenberg et al., 1993). Tir was originally incorrectly identified as a host cell tyrosine-phosphorylated heat shock protein-90 and was later identified as bacterial in origin, rather than mammalian (Kenny et al., 1997). Tir is inserted into the plasma membrane of the host epithelial cell, which requires the type III secretion system (T3SS) and both EspA and EspB. EPEC strains with deficient EspA and EspB do not translocate Tir when in culture with HeLa cells (Kenny and Finlay, 1995). An external small extracellular loop of Tir attaches to the intimin remaining in the outer membrane of the bacterium (Kenny et al., 1997). Once this tight adherence is formed, the epithelial cell shape changes and forms a pedestal beneath the bound bacteria. The binding of Tir and intimin allows for tighter adherence to the host epithelial cell, but other adhesins can also play a role in the binding of EPEC.

Adhesins other than intimin can play varying roles in adhesion of EPEC to epithelial cells and to each other when forming a biofilm. Bundle forming pilus, the type IV fimbria in
typical EPEC, is associated with early adherence to intestinal epithelial cells. Bundle forming pilus (BFP) is also important for the localized adherence pattern (LA) shown in HEp-2 cells and other cell lines (Cravioto et al., 1991). RelA, a protein associated with EPEC response to low nutrition, also plays a role in adherence associated with BFP and intimin (Spira et al., 2014). A strain of EPEC associated with porcine diarrhea produces a porcine attaching and effacing associated-protein \((paa)\) and loss of \(paa\) leads to impeded adherence of EPEC in pigs (An et al., 1999). The fimbrial adhesin subunit \(fimA\) in atypical EPEC is also associated with inducing a diffuse adherence (DA) pattern on HeLa cells (Hernandes et al., 2013). Other fimbrial adhesins present in both commensal and pathogenic \(E. coli\) can be found in EPEC but their role in pathogenicity is undetermined (Hernandes et al., 2011). Lastly, the \(LifA/efa1\) gene encodes a protein that contributes to epithelial cell adherence but not A/E lesion formation of EPEC, EHEC, and STEC (Badea et al., 2003). The ability of EPEC to adhere to each other and produce biofilm is associated with EspA, BFP, and other adhesins. Genes most highly associated with biofilm forming atypical EPEC are type I fimbriae \(fimA/fimH\) genes and curli genes \((csgA and crl)\) (Nascimento et al., 2014).

EPEC’s adherence requires additional components of the T3SS. The EscN protein is an ATPase that provides the energy to run the machinery of the T3SS (Gauthier et al., 2003). EscA/EscO (previously Orf15) is necessary for formation of T3SS in EPEC, EHEC, and \(C.\) \(rodentium\) (Sal-Man et al., 2012). EscF, found in LEE locus 4, forms the initial needle and is required for T3SS formation (Sekiya et al., 2001). EPEC with loss of EscF cannot form the A/E lesion when inoculated into HeLa cell culture (Sekiya et al., 2001). The EspA proteins form a filamentous sheath that elongates the EscF needle in the extracellular space (Knutton
et al., 1998; Ogino et al., 2006). Together, the T3SS and the adhesins of EPEC provide EPEC the ability to adhere to the intestinal epithelial cell.
**EPEC alters intestinal permeability and absorption**

Once adhered to the intestinal epithelial cell, effectors are secreted from EPEC through the T3SS and into the cell. These effectors lead to multiple alterations of the normal cell function, including changes to permeability and absorption. EPEC causes decreased trans-epithelial electrical resistance (TEER) when EPEC is inoculated on an epithelial cell monolayer due to increased permeability (Spitz et al., 1995; Yuhan et al., 1997). The decrease in TEER is associated with alteration in tight junction proteins, occludin, claudin-1, and zonula occludens (ZO-1). In particular, EPEC infection causes occludin relocation from the membrane and punctate staining in cytoplasmic compartments was noted with two-dimensional immunofluorescence (Muza-Moons et al., 2004; Simonovic et al., 2000). Immunofluorescence for claudin showed that claudin retained peripheral membrane location but became disorganized post EPEC infection (Muza-Moons et al., 2004). Lastly, ZO-1 staining became lost in some areas of the membrane post infection (Muza-Moons et al., 2004). EPEC infection also decreases the normal interactions between claudin-1 and occludin and between occludin and ZO-1 (Muza-Moons et al., 2004).

Re-localization of tight junction proteins is due in part to effector protein EspF (McNamara et al., 2001; Muza-Moons et al., 2004). Mutation of EspF diminishes re-localization of tight junction proteins (McNamara et al., 2001). Infection of T-84 cell monolayers with espF mutants does not yield the decreased TEER that wild type EPEC causes (McNamara et al., 2001; Muza-Moons et al., 2004). Furthermore, complementation of the espF mutant with a plasmid containing espF returns EPEC’s ability to decrease TEER (McNamara et al., 2001). The effects of EPEC on TEER were shown to be unassociated with
cell death by measuring release of lactate dehydrogenase from T84 cells that were uninfected, infected with wild type, and infected with espF mutant (McNamara et al., 2001). EPEC associated re-localization of claudin-1 and occludin, with the addition of caveolin-1, also occurs in vivo in a mouse model infected by the typical EPEC strain E2348/69 (Zhang et al., 2012).

EspG and EspG2 also work to internalize the tight junction protein, occludin (Glotfelty et al., 2014). Using MDCK and Caco-2 cells, EPEC infection induced abnormal localization with loss of staining at the periphery of the cells for ZO-1, claudin-1, and claudin-4. This re-localization did not occur when a ΔEspG1/G2 mutant was used, indicating the necessity of EspG1 and EspG2 in perturbation of tight junction proteins (Glotfelty et al., 2014). In addition, EPEC infection inhibits restoration of tight junctions after calcium chelation resulting in longer effect on permeability and longer decrease in TEER. EspG1 and EspG2 associated re-localization of tight junctions is linked with their effect on microtubules.

Adherence and microvilli effacement caused by EPEC leads to loss of absorptive surface in the gastrointestinal tract. In addition, effector proteins can directly inhibit transporter proteins on the intestinal epithelial cell, also leading to decreased absorption (Dean et al., 2006). EspF binds to SGLT1, the Na+/glucose transporter, and Na+/H+ exchanger (NHE3) inhibiting the normal electrolyte and glucose absorption of the intestine (Hodges et al., 2008). EspG and EspG2 enter the cell through the T3SS and decrease the activity of down-regulated in adenoma (DRA) anion exchanger leading to decreased chloride and hydroxide exchange (Gill et al., 2007; Glotfelty and Hecht, 2012). Within 60 minutes of infection, EPEC causes inhibition of chloride and hydroxide exchange in T84 and Caco-2
cells (Gill et al., 2007). Immunofluorescence studies showed that the decrease in activity of the DRA anion exchanger occurs due to relocalization of DRA from the plasma membrane to intracellular compartments (Gill et al., 2007).
**EPEC effectors associated with cell death**

EPEC can directly lead to loss of epithelial cells lining the gastrointestinal tract. In addition to its effect on phagocytosis and tight junctions, EspF also targets the mitochondria, where it alters mitochondrial membrane potential, leading to release of cytochrome c, caspase cleavage, and cell death (Kenny and Jepson, 2000; Nagai et al., 2005; Nougayrede and Donnenberg, 2004). EspF was shown to enter the mitochondria, by co-localization with mitochondrial immunofluorescence markers in HeLa cells, immunoprecipitation, and fractionation (Nagai et al., 2005; Nougayrede and Donnenberg, 2004). EspF also moves into the nucleus in late-stage of the infection where it causes re-localization of nucleolin from the nucleus to the cytoplasm and also alters U8 small nucleolar RNA location, both of which are associated with ribosomal biogenesis (Dean et al., 2010). Additionally, infection of mice with *C. rodentium* with mutated EspF showed decreased mortality, decreased diarrhea, and decreased intestinal wall edema (Nagai et al., 2005). Overall, this indicates a close association between EspF and pathogenesis of EPEC. Similarly to EspF, Mitochondrial associated protein (map) alters mitochondrial membrane permeability causing release of cytochrome c and caspase cleavage. Map and EspF share a similar mitochondrial targeted sequence (Kenny and Jepson, 2000; Nagai et al., 2005). Loss of epithelial cells lining the intestine is a possible mechanism for disease due to EPEC in *vivo*, however it is infrequently considered a mechanism for increased permeability in *in vitro* studies.
EPEC effectors that modulate the innate immune response

EPEC mechanisms of resistance to the innate immune response are not fully understood. For instance, one proposed mechanism of resistance to antimicrobial peptides (AMP) is bacterial induced proteolysis of the AMP’s. Omptins (ompT) are a class of outer membrane proteases found on *E. coli* (Thomassin et al., 2012a; Thomassin et al., 2012b). Interestingly, the gene that encodes for ompT is present in both EPEC and enterohemorrhagic *E. coli* (EHEC), but ompT expression is higher in EHEC than in EPEC (Thomassin et al., 2012a; Thomassin et al., 2012b). Additionally, degradation of the cathelicidin, LL-37, is more rapid and more complete when inoculated with EHEC, as compared to EPEC (Thomassin et al., 2012a). This difference in expression and activity of the omptin in EHEC and EPEC is thought to be associated with bacterial evolutionary response to the different concentrations of AMP’s in the small intestine, where EPEC resides, versus the large intestine, where EHEC resides. Further research regarding the inhibitory responses of EPEC to antimicrobial peptides is needed and could elucidate a possible mechanism for disease and susceptibility to colonization in the intestine.

Translocated intimin receptor (Tir) can inhibit innate immune responses in addition to its role in attachment of EPEC to the intestinal epithelial cell. Tir inhibits the NF-κB signaling pathway by inhibiting TNFα induction of IKK (Ruchaud-Sparagano et al., 2011). Release of NF-κB from IκB was inhibited when a Tir plasmid construct was transfected into HeLa cells *in vitro* (Ruchaud-Sparagano et al., 2011). Tir also contains two domains that replicate the immunoreceptor tyrosine-based inhibitory motif (ITIM) associated with suppression of host immune response. Tir can bind to host tyrosine phosphatases, SHP-1 and
SHP-2 (Yan et al., 2013; Yan et al., 2012). ITIMs and SHP’s are associated with inhibition of immune responses. EPEC infection or transfection of Tir in vitro leads to enhanced binding of SHP-1 or SHP-2 to TRAF6, a mediator of the TLR2-TLR4-MyD88 pathway (Yan et al., 2013; Yan et al., 2012). Binding of SHP-1 or SHP-2 to TRAF6 stabilizes TRAF6 and leads to decreased ubiquitination (Yan et al., 2013; Yan et al., 2012). Decreased ubiquitination leads to decreased activation of an NF-κB regulator (TAK-1), thus decreasing active NF-κB (Yan et al., 2013; Yan et al., 2012). Infection of macrophages in vitro (RAW264.7 or mouse primary peritoneal macrophages) with the EPEC strain, JPN15ΔTir (lacks Tir), causes higher expression of TNF and IL-6 as compared to wild type (Yan et al., 2012). This effect of Tir on TNF and IL-6 production was confirmed in vivo using the C. rodentium mouse model of EPEC. Tir, like many other effector proteins of EPEC, modulates host pathways, including the innate immune response.

One of the most studied strategies used by EPEC to modulate the innate immune response is subverting the NF-κB pathway. There are numerous non-LEE effectors (Nle) that affect the NF-κB pathway (Table 2). NleE1 suppresses the activity of NF-κB via suppression of multiple pathways (TNF receptor, TRAF2 and 5, MyD88, and TLR pathways) (Ruchaud-Sparagano et al., 2011; Zhang et al., 2011). NleE specifically acts as a methyltransferase and modifies the cysteine in host Tab2/3 (ubiquitin-chain sensory proteins) leading to dysfunctional ubiquitination of IκB and loss of NF-κB signaling (Nadler et al., 2010; Zhang et al., 2011). Transfection of NleE into HeLa cells caused loss of p65 (NF-κB subunit) nuclear translocation after IL-1β stimulation (Vossenkamper et al., 2010). NleE also reduces IL-8, TNFα, and IL-6 secretion from dendritic cells in culture. This study also showed that
NleE is able to translocate through an intact epithelial cell (T84) monolayer on a transwell to exert its effect on subepithelial dendritic cells (Vossenkamper et al., 2010). NleE and NleB stabilize IκB leading to decreased NF-κB activity and decreased IL-8 expression in HeLa cells in vitro (Nadler et al., 2010). The role of NleB in inhibition of the immune system has been shown in vitro using HeLa cells infected with C. rodentium. They found that NleB decreased TRAF2 ubiquitination leading to decreased NF-κB activation downstream (Gao et al., 2013). They further elucidated that NleB immunoprecipitated with GAPDH, mediated O-glcNAcylation of GAPDH, and disrupted GAPDH-TRAF2 function, all leading to NF-κB inhibition (Gao et al., 2013). NleH1 and NleH2 are Ser/Thr protein kinases found in EHEC, EPEC, and C. rodentium (Gao et al., 2009). They both bind directly to the ribosomal protein S3 (RPS3), as shown with immunoprecipitation of EPEC infected HeLa cell lysates and bimolecular fluorescence complementation (Gao et al., 2009). The interaction of NleH1 with RPS3 causes reduced nuclear presence of RPS3 in 293T cells (human embryonic kidney cells) and HeLa cells (Gao et al., 2009). Although NleH1 did not alter the nuclear presence of NF-κB subunit p65, transfection of NleH1 into 293T cells decreased TNFα responsive NF-κB activity to 45% of normal levels (Gao et al., 2009). Another study found that NleH1 and NleH2 decreased NF-κB activity downstream of IKKβ, due to inhibition of ubiquitination of IκBα in HEK293T cells transfected with NleH1 or NleH2 (Royan et al., 2010). In vivo studies also indicate that NleH1 and NleH2 suppress NF-κB activity; deletion of NleH1 and NleH2 caused increased KC (mouse homologue of IL-8) levels in streptomycin treated mice, as compared to wild type EPEC (Royan et al., 2010).
Effector molecules of EPEC often subvert the NF-κB pathway by affecting the subunit p65. The effector protein, NleC, inhibits NF-κB and reduces inflammatory cytokine secretion (Hodgson et al., 2015; Muhlen et al., 2011; Pearson et al., 2013; Sham et al., 2011; Yen et al., 2010). Infection with ΔNleC EPEC in Caco-2 and HT-29 cells induces higher IL-8 secretion than wild type (Sham et al., 2011). Not only does NleC inhibit NF-κB, but it also inhibits p38 MAP kinase that is also a signaling pathway of immune responses (Sham et al., 2011). Studies have also shown that infection of C57BL/6 mice with ΔNleC C. rodentium caused increased inflammatory cell infiltrates, increased edema, increased hyperplasia, and increased chemokine response without increasing the colonization of C. rodentium (Hodgson et al., 2015; Sham et al., 2011). Additional studies regarding NleC showed that NleC cleaves the NF-κB subunit p65, leading to its ubiquitination and proteosomal degradation in the cytosol (Yen et al., 2010). Reduction of p65 was noted in HeLa cells infected with EPEC, whereas there was no reduction when cells were infected with a ΔNleC strain (Yen et al., 2010). The cleavage of p65 was also shown in HEK293T cells and mouse primary colonic epithelial cells infected with C. rodentium and in Caco-2 cells infected with EPEC (Hodgson et al., 2015). In addition to cleaving p65, thus leading to degradation, NleC also binds to RPS3 (similar to NleH1 and NleH2) and decreases its nuclear translocation (Hodgson et al., 2015). Research indicates that NleC is specific to p65; however, there is conflicting data regarding its ability to cleave p50 (another Rel subunit of NF-κB) (Hodgson et al., 2015; Muhlen et al., 2011). NleC acts as an inhibitor of NF-κB by degrading the subunit p65 in a time dependent manner (Pearson et al., 2013). All of the above effector proteins likely have
the capacity to affect the immune response alone but also often induce an additive effect on suppression of NF-κB.

The innate immune response associated with inflammasome construction may also be inhibited by EPEC. The inflammasome response is associated with recognition of flagellar fragments, LPS, and the TTSS, leading to secretion of IL-1β (Knodler et al., 2014; Nordlander et al., 2014). The effector protein, NleA, decreases IL-1β by inhibiting caspase-1 activation and ASC-containing NLRP3 inflammasomes (Yen et al., 2015). Research on NleA was performed using cultures of LPS-primed differentiated THP-1 cells (monocytic leukemia cell line), which may not recapitulate the role of NleA in inhibition of inflammasomes in epithelial cells or inflammatory cells after infection with EPEC.

The modulation of the innate immune response by EPEC is not always directed towards immune suppression. Few Esp’s are associated with an increase in inflammatory mediators. The Wxxxx effector, EspT, induces macrophages (U937) to increase levels of COX-2 and produce IL-1β and IL-8 in an NF-κB and Rac1 dependent manner (Raymond et al., 2011). Although this was predominately shown in vitro, ΔEspT C. rodentium infection in C57/BL-6 generated decreased levels of KC, the mouse cytokine similar in structure and function to IL-8 (Raymond et al., 2011). The NleE2 effector leads to activation of NF-κB and high nuclear levels of p65; however, this suppression is inhibited by the NF-κB suppressor, Tir (Ruchaud-Sparagano et al., 2011). The effector protein, NleF, also leads to degradation of IκBα, leading to release of NF-κB, nuclear translocation of p65, and upregulation of IL-8 (Pallett et al., 2014). Although NleF also binds to caspase-4, -8, and -9, its interaction with caspases was not required for p65 nuclear translocation (Pallett et al., 2014).
Host defense against EPEC

There are multiple non-specific innate immune defense mechanisms in the intestinal tract that are important for protection against bacteria and other pathogenic organisms. These mechanisms include mucus production and creation of a mucus barrier overlying the epithelium, glycocalyx barrier also overlying the epithelium, antimicrobial peptides, the epithelial cells themselves, and intestinal epithelial cell receptors. Each of these mechanisms is associated with host innate immune response to EPEC infection. The most studied innate immune responses to EPEC include: pattern recognition receptors (PRR’s) and other receptors, signaling pathways downstream of PRR’s, cytokine production, and epithelial cells.

Antimicrobial peptides are secreted from Paneth cells and epithelial cells in the intestine and act as antibacterial substances. Antimicrobial peptides (AMP’s) can cause bacterial agglutination and cell lysis (Vaishnava et al., 2008). They are likely to be highest in concentration near the epithelial surface of the intestine, thus exerting antibacterial effects on pathogenic bacteria, while not affecting the luminal microflora (Thomassin et al., 2012b). Antimicrobial peptides are produced in response to bacterial sensing and signaling via MyD88 receptors (Vaishnava et al., 2008). The antimicrobial peptide, human β-defensin 1, is constitutively expressed. Whereas human β-defensin 2 is expressed in response to IL-1, enteroinvasive E. coli, and Salmonella as shown by in vitro studies using Caco-2 cells and HT-29 cells (derived from human colorectal adenocarcinoma) (O'Neil et al., 1999). This responsive expression of human β-defensin 2 has been shown by immunohistochemistry detection in surface and crypt epithelium from human patients with ulcerative colitis and not
in normal sections (O'Neil et al., 1999). The production of β-defensins and other AMP’s are regulated by IL-22, which is produced by inflammatory cells in the lamina propria. Interleukin 22 binds to the IL-22 receptor present on epithelial cells leading to induction of Stat and production of β-defensins (Wolk et al., 2004; Zheng et al., 2008). The importance of Stat and production of AMP’s has been recently shown in the EPEC infection model of C. rodentium. Infection of Stat3 -/- mice with C. rodentium leads to more severe colitis and abrogation of the AMP, RegIIIγ (Wittkopf et al., 2015). Differentiated surface epithelial cells of the colon express another antimicrobial peptide, cathelicidin (LL-37) (Hase et al., 2002). Cathelicidin mRNA expression increases with the duration of cell culture (Caco-2, HCA-7, and HT-29 cells) and in these cells after differentiation by butyrate (Hase et al., 2002). In contrast to β-defensin 2, cathelicidin is not upregulated by interleukins or other pro-inflammatory mediators; however, it is upregulated by invasive bacteria like Salmonella and enteroinvasive E. coli (Hase et al., 2002). Lastly, the cathelicidin-related antimicrobial mouse protein (mCRAMP) is important in the innate immune response to C. rodentium (Iimura et al., 2005).

Pattern recognition receptors (PRR’s) also play an important role in innate immune responses to EPEC. PRR’s are found along the cell surface of epithelial cells lining the intestine as well as within the cytoplasm. In particular, the toll like receptor 5 (TLR 5) recognizes the flagella of EPEC and once bound to the TLR 5, downstream signaling leads to MAP kinase activation and release of NF-κB from IκB (Figure 1). NF-κB is translocated to the nucleus where it transcribes cytokines, like IL-8, as well as antimicrobial peptides. It has been shown that non-flagellated C. rodentium and EPEC cause decreased production of IL-8,
indicating the importance of the flagella for the innate immune response. This was shown using *in vitro* (HEp-2 cells) and *in vivo* studies (mice) with non-flagellated EPEC (Edwards et al., 2011; Khan et al., 2008). Furthermore, infection of HT-29 cells with EPEC led to increased TLR5 on the cell surface, as opposed to within intracellular compartments in control, uninfected cells (Salazar-Gonzalez and Navarro-Garcia, 2011). The receptor, TLR4, recognizes lipopolysaccharide (LPS) on the surface of EPEC, also leading to NF-κB activation and transcription of inflammatory cytokines (Khan et al., 2006). Lamina propria fibroblasts recognize LPS from EPEC, as well, leading to production of IL1α, IL1β, IL6, IL8, and TNFα (Chakravortty and Kumar, 1999). The interleukin receptor 7 (IL-7R) is present on epithelial cells and EPEC leads to up-regulation of IL-7R mRNA in T84 cells (Yamada et al., 1997). Although up-regulation of IL7R and subsequent production of IL-8 were linked to EPEC infection *in vitro*, it is not clear what bacterial protein induces this increase in IL7R. Receptors like PRR’s on membranes of both epithelial cells and the lymphocytes of the intestine are closely associated with the innate immune response to EPEC.

Intracellular PRR’s and inflammasomes are also important for recognition of EPEC and downstream inflammatory responses. The nucleotide binding and oligomerization domain (NOD) like receptor, NLRC4, is present in the crypt intestinal epithelial cells (Nordlander et al., 2014). NLRC4 recognizes fragments of flagella and components of the EPEC TTSS, leading to inflammasome production and release of IL-18 and IL-1β (Nordlander et al., 2014). The importance of NLRC4 in host response to EPEC was shown using the *C. rodentium* model. NLRC4 knockout mice developed more severe colitis, increased bacterial colonization in the colon, and reduced “steady state” IL-18 after infection
with *C. rodentium* (Nordlander et al., 2014). Furthermore, NLRC4 knock out led to decreased innate immune response and increased adaptive immune response, postulated to be in response to defective innate immunity (Nordlander et al., 2014). LPS is also recognized by caspase-4 and a noncanonical inflammasome leading to IL-18 production (Knodler et al., 2014). Although caspase-4 predominately recognizes intracellular bacterial components, caspase-4 dependent IL-18 was produced when EPEC was added to polarized human colonic cells *in vitro* (Knodler et al., 2014). Infection of cultured bone marrow derived macrophages with *C. rodentium* showed that NLRP3 inflammasomes, caspase-11, and TIR-domain-containing adaptor-inducing interferon-β (TRIF) were necessary for secretion of IL-1β and IL-18 (Rathinam et al., 2012). These studies showed the importance of inflammasome response to Gram-negative attaching and effacing bacteria like EPEC and *C. rodentium*.

Signaling pathways downstream of EPEC recognition by the aforementioned PRR’s are instrumental in producing the innate immune response to EPEC. As previously mentioned, NF-κB plays a large role in transcription of inflammatory mediators. EPEC, but not non-pathogenic *E. coli* or purified LPS, activates NF-κB in T84 human colonic epithelial cells (Savkovic et al., 1997). In this cell line, they showed that EPEC-activation of NF-κB leads to transcription of IL-8, which is often measured as a substitute for NF-κB activity (Savkovic et al., 1997). To be activated, NF-κB needs to be released from its inhibitory binding protein, IκB. The IκB kinase (IKK) phosphorylates IκB to release NF-κB (Gao and Hardwidge, 2011). Ubiquitination of IκB also leads to proteasome degradation of IκB and release of NF-κB (Royan et al., 2010). Atypical PKCζ, activated by EPEC, can also activate IKKα/β to release NF-κB from inhibitory IκB (Savkovic et al., 2003). An additional NF-κB
subunit, ribosomal protein S3 (RPS3) is translocated into the nucleus with NF-κB and targets NF-κB to specific promoters to control which genes are transcribed (Gao and Hardwidge, 2011). The NF-κB pathway is an important signaling pathway connecting the recognition of pathogens via PRR’s to transcription of inflammatory mediators (e.g. IL-8).

The host innate immune response to EPEC or *C. rodentium* leads to production and release of inflammatory mediators. These inflammatory mediators are often transcribed and secreted downstream from the previously discussed PRR’s. The chemokine, IL-8, produced downstream of TLR 5 and other PRR’s, leads to chemotaxis of neutrophils to the site of infection. In addition, the CXC chemokine receptor, CXCR3, leads to recruitment of neutrophils and killing of *C. rodentium* in mice (Spehlmann et al., 2009). The immune system must constantly balance between promoting inflammation and limiting it. Interleukin-10 is associated with balancing mucosal immune response. Mice deficient in IL-10 (IL-10 −/−) have increased mortality and increased inflammation due to *C. rodentium* infection (Krause et al., 2015). Overall, IL-10 and the macrophages that produce it are necessary to limit the inflammatory response to *C. rodentium*. Inflammatory cytokines, like IL-8, IL-10, IL-22, and others, play an important role in host immune response to EPEC or *C. rodentium*.
Experimental and naturally-occurring animal models of EPEC

Mice

The mouse model remains an important animal model for the study of EPEC; however, mice are not a natural host for EPEC. Comparably to EPEC, the bacteria, *Citrobacter rodentium* adheres to the epithelial cells via intimin, produces a type III secretion system, and contains the locus of enterocyte effacement (Deng et al., 2001). These similarities between EPEC and *C. rodentium* allow for its use as a bacterial model and its use as a model has been extensively reviewed (Borenshtein et al., 2008; Collins et al., 2014b). However, *C. rodentium* causes transmissible murine crypt/colonic hyperplasia or colitis. The colonic colonization of *C. rodentium* does not recapitulate disease in children where the small intestine is more frequently affected by EPEC (Luperchio and Schauer, 2001). The susceptibility of mice to *C. rodentium* infection and colitis is also dependent on mouse strain; some strains (C3H/HeJ, FVB, AKR/J) have fatal disease and some (C57BL/6, BALB/c, NIH Swiss) are more resistant to colitis (Borenshtein et al., 2007; Vallance et al., 2003). Therefore, researchers must carefully select a mouse strain for experimental infection.

Although disparities exist between disease due to EPEC in children and disease due to *C. rodentium* in mice, it has been a useful model for elucidating possible host factors associated with susceptibility and for testing potential therapeutics. The susceptibility and hyperplastic nature of *C. rodentium* infection are attributed to its effects on R-spondin, the Wnt/β-catenin signaling pathway, and epithelial differentiation (Papapietro et al., 2013). The role of R-spondin in the hyperplastic crypts of *C. rodentium* infected mice may also occur in EPEC infection; however, the stem cells of the small intestine have varying expression as
compared to stem cells of the colon (Cramer et al., 2015). Identification of potential therapeutics using the C. rodentium model include: vasoactive intestinal peptide, properdin, and multiple probiotics (Chen et al., 2005; Collins et al., 2012; Collins et al., 2014a; Conlin et al., 2009; D'Arienzo et al., 2006; Jain et al., 2015; Johnson-Henry et al., 2005; Mackos et al., 2013).

Attempts to infect mice with EPEC isolates have met with variable results. Mice have no morbidity associated with EPEC infection, low numbers of EPEC are shed in feces, and EPEC colonization is equal to nonpathogenic E. coli (Klapproth et al., 2005; Mundy et al., 2006). Experimental infection of mice with EPEC, similarly to C. rodentium infection, shows colonization in the large intestine, although in rare studies EPEC colonized both the small and large intestine (Savkovic et al., 2005; Shifflett et al., 2005). In 6-8 week old C57BL/6J mice, typical and Δbfp EPEC colonized the small and large intestine at 3 days post-infection, caused microvillus effacement, and bacteria were highest in the cecum (Savkovic et al., 2005). This EPEC mouse model has been used to confirm in vitro results but has also been debated regarding its translatability (Mundy et al., 2006). The inhibition/internalization of Cl-/OH- exchanger, Slc26a3 or Down-regulated in adenoma, associated with EPEC inoculation in Caco-2 cells was corroborated by infection studies in C57BL/6J mice (Gill et al., 2007). Similarly using the mouse model, the role of espF in tight junction barrier disruption was shown to occur in vitro and in vivo (Shifflett et al., 2005). One issue with mouse models of EPEC infection is that they are predominantly performed on adult mice. Recently utilization of neonatal mice in experimental infection with EPEC demonstrates colonization of the small intestine, indicating neonatal mice may serve as a more appropriate model (Dupont et al., 2006).
However, neonatal mice, like adult mice, still lack clinical signs of diarrhea after EPEC infection (Dupont et al., 2016; Savkovic et al., 2005).

**Rabbits**

The rabbit is one of the most frequently used animal model for EPEC and rabbits can naturally acquire EPEC infection. The rabbit strains of EPEC (e.g. rabbit EPEC (REPEC) and rabbit-derived *E. coli* (RDEC)) have been extensively studied and characterized (Cantey and Blake, 1977; Marches et al., 2000). Rabbit EPEC adhere most proficiently to the enterocytes lining the cecum and colon. Rabbit EPEC strains differ from human EPEC in their adherence patterns. Whereas typical EPEC initial adherence is associated with bundle forming pilus, REPEC Adherence Factor /Rabbit 1 (AF/R1) or AF/R2 is associated with adherence and cytopathic effects (Nougayrède et al., 1999; Ryu et al., 2001; Wolf et al., 1988). Rabbit EPEC has also been shown to bind to M cells overlying the Peyer’s patches, which has not been demonstrated in EPEC infection in human hosts (Inman and Cantey, 1984). The rabbit animal model often uses its own strains of EPEC but EPEC strains from other animal infections have also been investigated in rabbits (Goffaux et al., 1997). A common methodology used with rabbits is the rabbit ileal loop model or removable intestinal tie-adult rabbit diarrhea (RITARD) model (Mynott et al., 1991; Sampaio et al., 2014). This model uses multiple sections of the intestine, which allows for numerous experiments in a single animal.

There are some more translatable benefits to using the rabbit model of EPEC infection. Comparably to EPEC infection in humans, rabbit EPEC strains predominantly
infect suckling and weanling rabbits. Indeed, some strains of EPEC are associated with infection in weanling rabbits, while others are more often identified in suckling rabbits with diarrhea (Peeters et al., 1984; Peeters et al., 1988). Furthermore, suckling was partially protective against rabbit EPEC strain E22 (O103) infection (Gallois et al., 2008; Gallois et al., 2007; Skrivanova et al., 2008). This finding concurs with the protective benefits of breast-feeding identified in infants (Blake et al., 1993; Loureiro et al., 1998). Rabbit models have allowed for the identification of novel adherence factors and transcription regulators (Srikhanta et al., 2013a; Srikhanta et al., 2013b). The regulators in rabbit EPEC are similar to a regulator in C. rodentium and one in typical EPEC from humans (Srikhanta et al., 2013a).

Histopathology following rabbit EPEC infection is similar to lesions in other animal models and children. Lesions in the small intestine, cecum, and colon include: atrophy of the villi, flattened epithelium, epithelial ulceration, heterophilic (rabbit neutrophil-like cell) inflammation in the lamina propria, and loss of microvilli (Cantey and Blake, 1977; Crane et al., 2007; Marches et al., 2000; Milon et al., 1999; Takeuchi et al., 1978). New Zealand white rabbits from commercial vendors had an EPEC prevalence of 4%, including a serotype previously associated with diarrhea in rabbits (Swennes et al., 2013).

However beneficial studies on rabbit EPEC have been and continue to be, there are, as with any animal model, a few limitations. Rabbit digestion is very different from human digestion; they are herbivores and cecal fermenters. Rabbit EPEC often colonizes the ileum and cecum, although the ileum is likely similar to other mammals, the cecal resident bacteria and environmental milieu is different. Therefore rabbit EPEC may have additional genes and
functions to survive this environment that would not translate to functions in EPEC in children.

*Cattle*

The role of EPEC infection in calves has been recently reviewed by Kolenda et al. (Kolenda et al., 2015). Both healthy and diarrheic calves shed attaching and effacing *E. coli* (AEEC) and represent a reservoir for AEEC (EPEC, EHEC, and STEC) infections in humans, including O157:H7 (Kolenda et al., 2015; Moxley and Smith, 2010). Although normal healthy calves shed EHEC, serotypes carried by calves are associated with diarrhea, including O5, O26, O111, and O118 (Bardiau et al., 2009; Stordeur et al., 2000). Due to the risk of calves acting as reservoirs of EPEC, studies have been performed to determine the antimicrobial resistance in EPEC isolated from calves. Antimicrobial resistance is present in AEEC in calves, lambs, and kids, with EPEC isolates often being resistant to sulfamethoxazole, erythromycin, streptomycin, and tetracycline (Medina et al., 2011).

Early studies of diarrheagenic *E. coli* in cattle focused on the epidemiology of DEC in herds. An atypical *E. coli*, designated S102-9, was identified in calves with generalized dysentery (Hall et al., 1985). This *E. coli* was isolated, shown to be attaching and effacing, and was additionally used to experimentally infect gnotobiotic calves (Chanter et al., 1984; Hall et al., 1985; Moxley and Francis, 1986). In the experimental infection, out of all the multiple infectious etiologies used, only the *E. coli* S102-9 caused disease. Although these studies occurred before intimin was discovered, they were able to identify attachment and effacement *in vitro* and *in vivo*. Few studies have used a calf model of DEC infection as a
translational model of DEC in children. However, research on DEC in calves was instrumental in discovering the protective benefits of colostrum and the potential therapeutic use of bacteriophages (Logan et al., 1977; Smith et al., 1987). In the more epidemiologic studies, calves with and without diarrhea have been compared for presence of lesions in the gastrointestinal tract and presence of DEC. These studies often identified a higher prevalence of EPEC (as defined by PCR amplification of eae and lack of shiga-like toxins in bacteria) in healthy calves (China et al., 1998; Coura et al., 2015; Holland et al., 1999b). Furthermore, a study in Brazil found no association between the presence of different types of pathogenic E. coli and diarrhea (COURA et al., 2015).

Investigation of EPEC or attaching and effacing bacteria in calves with diarrhea have demonstrated histopathologic lesions similar to children infected with EPEC. Attaching and effacing bacteria have been identified in both small and large intestines of calves (Gunning et al., 2001; Hall et al., 1985). In one study, they confirmed that healthy calves shedding EPEC also contain the typical attaching and effacing (A/E) lesion (Pospischil et al., 1987). Histopathologic lesions noted in the small intestine of calves include: villus blunting, neutrophils in the lamina propria, and infrequent loss of epithelial cells at villus tips (Logan et al., 1977). Electron microscopy identified microvilli blunting in the colon of < 3-week-old calves with diarrhea and AEEC (Hall et al., 1985). It is important to note that prior to the 1990’s, studies did not differentiate attaching and effacing E. coli (AEEC) into either EPEC or EHEC. Therefore, the association of hemorrhagic lesions in the colon with AEEC was likely an association with EHEC rather than EPEC (Janke et al., 1990).
Diarrheagenic *E. coli*, while infrequently associated with diarrhea in calves, are likely contributing to disease in calves and zoonotic infection in children. It is likely that the AEEC may not act as an enteropathogen alone in calves, but contribute or worsen disease due to other pathogens. Elucidating the interactions between AEEC and other organisms is one manner in which calves can be further used as an animal model. Another benefit of the calf as a naturally-occurring animal model of AEEC is the age range of susceptibility to disease; calves are often affected by AEEC when very young (2 to 12 weeks old), comparable to the susceptibility of young children to EPEC associated diarrheal disease (Aidar et al., 2000; China et al., 1998; Hall et al., 1985). Future studies should also investigate the roles of DEC in malnutrition or reduced weight gain in calves as both a translational model to malnutrition in children and to improve health of calves in the meat and dairy industries.

Small ruminants

Small ruminant EPEC infections are not documented as much as for calves. In one retrospective study, only 2 lambs were identified with diarrhea and an attaching and effacing *E. coli*. Out of hundreds of *E. coli* cultured from lambs and kids with diarrhea, only 50 demonstrated the *eae* gene (Cid et al., 2001). As in other species, co-infections are common and coccidia and EPEC have been identified concurrently. In lambs, colostrum deprivation and infection with *Cryptosporidium parvum* led to increased shedding of EHEC serotype O157:H7 (La Ragione et al., 2006). Although DEC infection in lambs is less common than in calves, few research studies have been performed using the lamb as an animal model (Altmann and Mukkur, 1983; Gregory et al., 1983; Smith, 1976). Although research on the
association of aEPEC with disease in small ruminants is limited, there are multiple studies implicating both goats and sheep as sources or reservoirs of infection (Frohlicher et al., 2008; Krause et al., 2005; Orden et al., 2010). In sheep, the prevalence of attaching and effacing *E. coli* (*eae* positive) was determined to be 19% (Krause et al., 2005). Likewise, a more recent study demonstrated a 19% prevalence of EPEC in lambs, while no EPEC was identified in adults (Martins et al., 2016). EPEC in sheep has been confirmed to be diverse by pulsed-field gel electrophoresis (PFGE) and serotype (Frohlicher et al., 2008; Martins et al., 2016).

**Pigs**

Similar to calves, healthy and diarrheic pigs can shed EPEC. The A/E lesion was first identified in gnotobiotic piglets in 1969 (Staley et al., 1969). Porcine post-weaning diarrhea (PWD) is predominately associated with ETEC, but EPEC can also be cultured from piglets with diarrhea (An et al., 1999; Chapman et al., 2006; Malik et al., 2017). Porcine attaching and effacing associated-protein (encoded by *paa*) is associated with adhesion of EPEC in piglets, but is also associated with ETEC and was identified as an adhesin along with other fimbrial adhesins of ETEC (F4(K88), F5(K99), F6(987P), F41, F42, F165, F17 and F18) (An et al., 1999; Leclerc et al., 2007; Nagy and Fekete, 1999; Vidotto et al., 2013). Experimental infection of human EPEC in piglets lacking colostrum has produced disease. Affected piglets are often in the age range of 1 week to 6 weeks (Janke et al., 1989). Lesions in 7 pigs with EPEC were primarily found in the small intestine (Janke et al., 1989). Ultrastructurally, EPEC infection in pigs causes similar lesions as in children, including the A/E lesion, microvillus blunting, and pedestal formation (Malik et al., 2017). Gnotobiotic pigs have
been used in EPEC research with direct infection and in an ileal loop model (Moon et al., 1983). The porcine model has been evolving as an important model for other gastrointestinal diseases due to the translatability. Pigs have similar diets to humans, have similar gastrointestinal tracts, and are genetically diverse.

Dogs and cats

Investigation of DEC carriage in companion animals has demonstrated shedding of DEC from both dogs and cats. One study discovered a shared strain of EPEC between a healthy child and a 3-month-old puppy with diarrhea (Rodrigues et al., 2004a). A study performed in Brazil found that dogs are more likely to shed *E. coli* with virulence factors compared to cats (Puno-Sarmiento et al., 2013). Of the DEC found in dogs with diarrhea, 12/17 were *eae* positive (Puno-Sarmiento et al., 2013). A study to investigate the prevalence of DEC in 5-month-old, research-bred dogs was performed in 1999 (Holland et al., 1999a). The study found *eae + E. coli* (EPEC) in 12 of 52 dogs tested (23%) and the serotypes found were: O2 (4 strains), O63 (3 strains), and O15, O88, and O115 (1 each). Dogs in Brazil were also studied for prevalence of EPEC and EPEC was found in 19/146 dogs with diarrhea (13%) and 3/36 dogs without diarrhea (8%) (de Almeida et al., 2012; Nakazato et al., 2004). In a retrospective study, dogs with AEEC were between 7 and 8 weeks of age (Wales et al., 2005). Intestinal lesions in dogs and cats are often localized to the small intestine. Both typical EPEC and atypical EPEC have been shown to cause diarrhea in dogs 24 to 72 hours post infection (Gouveia et al., 2013). EPEC has also been shown to be prevalent in groups of stray dogs and coyotes in the Southwest of the United states and Mexico (Jay-Russell et al.,
A study in Belgium identified both atypical EPEC and typical EPEC in feces of dogs and cats, however, the study only assessed diarrheic animals, so no association of EPEC with diarrhea could be elucidated (Goffaux et al., 2000).

Cats represent a possible reservoir and source of infection for aEPEC. Atypical EPEC can be cultured from the feces of cats with and without diarrhea (Goffaux et al., 2000; Morato et al., 2009). Even prior to the discovery of eae, serotypes suspected to be pathogenic E. coli were found in normal feline feces (Mian, 1959). In 1987, a study in Germany investigating EPEC as a pathogen in both calves and cats with diarrhea identified attaching and effacing bacteria in the ileum and colon of one adult cat and one 8-week-old kitten (Pospischil et al., 1987). Although they identified attaching and effacing bacteria, they did not confirm that the bacteria were indeed EPEC (eae + E. coli). In Belgium, EPEC was identified in 3 cats with diarrhea (Goffaux et al., 2000). In that study, they were able to show that the EPEC contained other virulence factors, in addition to eae. Indeed, the EPEC found in 2 of the 3 cats contained bfp identifying the EPEC as typical EPEC, which is, in children in developing countries, the more prevalent type of EPEC. Larger epidemiologic studies have recognized a low prevalence of EPEC in cats with or without diarrhea. Studies investigating the prevalence of EPEC in cats as well as other species identified a fecal EPEC prevalence in cats of 4.7% to 6% in Brazil and 6.5% in Germany (Krause et al., 2005; Morato et al., 2009; Puno-Sarmiento et al., 2013). One study found 14 out of 300 cats ≥ 3 months (70 with diarrhea and 230 with no history of diarrhea) contained EPEC (Morato et al., 2009). Although they found a low prevalence, they postulated that cats may represent an important reservoir species for EPEC, particularly because they identified serotypes that are known
diarrheagenic EPEC in children. In another Brazilian study atypical EPEC prevalence was found to be 6%; EPEC was cultured from 3 out of 50 feline fecal samples (Puno-Sarmiento et al., 2013). A study in our laboratory discovered enteroadherent EPEC in the small intestine or colon of 9/50 (18%) weanling kittens that died due to generalized illness (Ghosh et al., 2013). *Enterococcus hirae*, an enteroadherent member of the normal microbiota, was not present in kittens with EPEC, indicating a competitive adherence between *E. hirae* and EPEC in kittens (Ghosh et al., 2013).

**Avian**

*Escherichia coli* found in chickens is termed avian pathogenic *E. coli* (APEC) and it does not share pathophysiology with EPEC. APEC is actually considered more similar to extraintestinal pathogenic *E. coli* infections in humans (Koga et al., 2015). In contrast, atypical EPEC has been identified in turkeys with poult enteritis mortality syndrome (Pakpinyo et al., 2002, 2003). In affected turkeys, there is evidence of attaching and effacing lesions. Additionally, experimental infection with coronavirus and EPEC caused clinical signs 4 days post infection that consisted of decreased water intake, anorexia, and depression (Pakpinyo et al., 2002, 2003).

Wild birds may play a role as reservoirs for EPEC or other DEC. Wild avian species are carriers of EPEC, with a prevalence of 12.6% (31/246) and a wide diversity in pulsotype and serotype of EPEC (Kobayashi et al., 2002). A study in Canada found 16% of *E. coli* cultured from fecal samples of wild birds contained *eae* (Chandran and Mazumder, 2014). EPEC was highest in hawks and ducks (Chandran and Mazumder, 2014). Atypical EPEC is
shed by normal pigeons, ducks, and chickens in India (Farooq et al., 2009). Avian species appear to be carriers of EPEC, but do not, in general, show signs of diarrhea.

Other animals

An array of other animals have been investigated for carriage of EPEC and to determine if EPEC is a cause of diarrhea. Attaching and effacing *E. coli* have been identified in cotton-top tamarins and SIV+ Macaques, but the role of AEEC or EPEC in causing diarrhea has not been elucidated (Mansfield et al., 2001). EPEC has been cultured from multiple primate species in a Minnesota Zoo at a prevalence of 13% (Clayton et al., 2014). An EPEC O111 strain from a child with diarrhea was able to form the A/E lesion on ileal epithelial cell explants cultured from 2 one-month-old foals (Batt et al., 1989). However, in that study, no direct causation of diarrhea in foals was determined for EPEC. In another study, EPEC was isolated from healthy and diarrheic foals, but no association with diarrhea was determined (Holland et al., 1996). Screening of wild cervids in Belgium found isolated EPEC and STEC in feces (Bardiau et al., 2010). Although the ability for EPEC to cause diarrhea in non-human hosts is debated, the carriage of EPEC occurs in a wide range of hosts.
Treatment and Prevention of EPEC

Within the past 20 years, research on EPEC has expanded to investigate therapeutics. Investigations have predominantly focused on the use of prebiotics, probiotics, and vaccination. Prebiotics stimulate growth or activity of certain members of the microbiota to improve host health (Gibson and Roberfroid, 1995). Oligosaccharides, in particular a phosphorylated mannanoligosaccharide (MOS), have been investigated for their ability to ameliorate diarrhea caused by EPEC in boxer dogs (Gouveia et al., 2013). MOS binds to mannose sites on bacteria, thus preventing adherence of bacteria to mannose receptors on intestinal epithelial cells. Dogs receiving MOS recovered faster and had improved fecal consistency, however, the negative control group had short duration of diarrhea as well (Gouveia et al., 2013). Another prebiotic, fucosyl-disaccharide, was shown to inhibit adhesion of some EPEC isolates, but not all. The mechanism of why adhesion of only some EPEC isolates was affected was not demonstrated (Becerra et al., 2015).

Probiotics have also been investigated to prevent adhesion of EPEC and disease. The probiotic, *E. coli* Nissle 1917, inhibits EPEC adhesion by adhering to intestinal epithelial cells itself via F1C fimbriae. In addition, it forms a barrier by interconnecting flagella between bacteria, thus further preventing EPEC adhesion (Kleta et al., 2014). *Lactobacillus* spp. have also been studied for their potential as probiotic treatments against EPEC (Mack et al., 1999; Michail and Abernathy, 2002). *Lactobacillus plantarum* was able to decrease adhesion of EPEC by upregulating mucin in an intestinal epithelial cell line (Mack et al., 1999).
Vaccination using *E. coli* antigens has been studied for prevention of extraintestinal pathogenic *E. coli* (ExPEC) and ETEC. A particular protein, SslE (secreted and surface-associated lipoprotein from *E. coli*) is conserved in many *E. coli* and is associated with mucin degradation (Nesta et al., 2014). Antibodies against SslE impeded ExPEC movement across a gel-mucin matrix and reduced the number of ETEC strains in an intestinal mouse model of infection and reduced uropathogenic *E. coli* (UPEC) in a transurethral infection mouse model (Nesta et al., 2014). Immunoglobulin against adhesin proteins has also been used in development of EPEC and EHEC vaccines (Li et al., 2013). Nanoparticles of O111 polysaccharide from LPS bound to a carrier protein inhibited adhesion of O111 serotyped ETEC in a rabbit model (Andrade et al., 2014). Unfortunately, this would only be effective against that O serotype and many serotypes are associated with ETEC and EPEC.

**Future Directions**

Many *in vitro* and *in vivo* studies have elucidated the roles of virulence effectors on adherence and pathogenicity of EPEC. The mechanisms of the A/E lesion and secretion of effector proteins have become better understood. It is now recognized that proteins entering the host epithelial cell through the EPEC T3SS affect tight junction, absorptive surface (microvilli), immune response, cytoskeletal arrangement, and cell death. Of these changes to the host, it is unknown which play(s) the most important role(s) in causing diarrhea. Establishing new models to study the *in vivo* pathogenesis of EPEC should elucidate mechanisms of differing susceptibility to EPEC-induced diarrhea. Research identifying members of the intestinal commensal bacteria that can limit adherence of EPEC indicates that the microbiome may be involved in host susceptibility to EPEC colonization and disease.
(Kleta et al., 2014; Mack et al., 1999). Future research will likely focus on the gut microbiome of children or animals infected with EPEC and may elucidate a particular pattern of diversity that could differentiate children with EPEC and diarrhea from asymptomatic children with EPEC. Determining the structure of microbiome communities could lead to novel prebiotic or probiotic treatments to drive the microbiome to a protective, beneficial structure.
Table 1. Virulence effectors of EPEC that influence adhesion to epithelial cells.

<table>
<thead>
<tr>
<th>EPEC effector</th>
<th>Role in adhesion</th>
</tr>
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<tbody>
<tr>
<td>BFP</td>
<td>Present in typical EPEC, bacteria-bacteria binding (microcolony formation) and lose adherence to epithelial cells</td>
</tr>
<tr>
<td>EscN</td>
<td>ATPase that provides energy for formation of the T3SS</td>
</tr>
<tr>
<td>EspA</td>
<td>Filamentous protein that is exerted by and expands the T3SS to the epithelial cell</td>
</tr>
<tr>
<td>EspD/ EspB</td>
<td>Forms the pore in the host epithelial cell into which EPEC can insert virulence factors</td>
</tr>
<tr>
<td>Tir</td>
<td>Translocated intimin receptor binds to intimin for tight adherence and development of the A/E lesion</td>
</tr>
<tr>
<td>Intimin</td>
<td>Binds to TIR for tight adherence</td>
</tr>
<tr>
<td>Paa</td>
<td>Encoded by porcine attaching and effacing-associated gene, similar to eae, important for early adherence of EPEC, ETEC, and EHEC in pigs</td>
</tr>
<tr>
<td>RelA</td>
<td>Regulator of expression of BFP and intimin</td>
</tr>
<tr>
<td>Efa1/LifA</td>
<td>Adherence factor in EHEC and EPEC, the presence of the gene is associated with diarrhea in humans</td>
</tr>
</tbody>
</table>
Table 2. Virulence effectors of EPEC that modulate the innate immune response. Multiple Esp’s and Nle’s suppress the innate immune response, primarily via suppression of NF-κB.

<table>
<thead>
<tr>
<th>EPEC effector</th>
<th>Role in innate immune response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tir</strong></td>
<td>Inhibits TNFα induction of IKK, thus inhibits release of NF-κ; increases SHP binding to TRAF6 leading to decreased ubiquitination of TRAF6, decreased activation of NF-κB</td>
</tr>
<tr>
<td><strong>NleE1</strong></td>
<td>Inactivates ubiquitin proteins in NFkB signalling; NleE inhibits both p65 nuclear translocation and IκBα degradation; suppresses TNFR, TRAF2, TRAF5, MyD88, and TLR pathways</td>
</tr>
<tr>
<td><strong>NleB</strong></td>
<td>Works with NleE in stabilizing IκB to limit NF-κB activation; O-glcNAcylates GAPDH, disrupts GAPDH-TRAF2 interaction, leading to NF-κB activation</td>
</tr>
<tr>
<td><strong>NleC</strong></td>
<td>Zinc metalloproteinase that cleaves NFkB p65 subunit; binds to RPS3 and decreases its nuclear translocation</td>
</tr>
<tr>
<td><strong>NleH (1 and 2)</strong></td>
<td>Bind to RPS3; ser/thr protein kinases; reduces NF-κB activation; inhibits ubiquitination of IκBα</td>
</tr>
<tr>
<td><strong>NleA</strong></td>
<td>Decreases IL-1β by inhibiting caspase-1 and NLRP3 inflammasome</td>
</tr>
</tbody>
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CHAPTER II

Association of enteropathogenic *Escherichia coli* with diarrhea-related mortality in foster age kittens

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Abstract

Diarrhea is responsible for the death of approximately 900,000 children per year worldwide. In children, enteropathogenic *E. coli* (EPEC) is a common cause of diarrhea and is associated with higher hazard of death. Non-human reservoirs of EPEC are limited and disease is poorly reproduced in experimental animal models. As in children, mortality in kittens is often attributed to diarrhea and we previously identified enteroadherent EPEC in intestinal tracts of deceased kittens. The purpose of this study was to determine the prevalence of EPEC in kittens and its association with diarrhea, diarrhea-related mortality, gastrointestinal pathology, and possible infection risk factors. Kittens with and without diarrhea were obtained from two shelter facilities. Based on fecal culture the prevalence of EPEC in kittens was 18%. In contrast, qPCR detected the presence of the gene for intimin (*eae*) in feces from 42% of kittens. EPEC strains were cultured from kittens with and without diarrhea. However, kittens that died or were euthanized due to diarrhea harbored significantly greater quantity of EPEC and fecal *eae* compared to kittens without diarrhea. Kittens diagnosed with EPEC had significantly greater severity of inflammatory infiltrates in the small intestine and colon and increased epithelial injury in the small intestine. When all kittens were examined on the basis of EPEC diagnosis, significant associations were observed between EPEC infection and subcutaneous fluid administration. These findings identify EPEC as a prevalent infection in kittens and implicate EPEC as a significant primary or contributing cause of intestinal inflammation, dehydration, and diarrhea-associated mortality in kittens.
Introduction

Diarrhea is responsible for the death of an estimated 900,000 children per year worldwide with the majority of mortality occurring in developing countries (Black et al., 2010; Kotloff et al., 2013; Liu et al., 2012). A recent Global Enteric Multicenter Study (GEMS) determined that diarrheal death of these children can be largely attributed to a mere handful of infectious agents (Kotloff et al., 2013). In particular, diarrhea caused by enteropathogenic E. coli (EPEC) is associated with a 2.6 fold higher hazard of death – the largest reported in the study (Kotloff et al., 2013). Enteropathogenic E. coli is responsible for over 81 million cases of diarrhea per year of which 17 million cases are diagnosed in children (Kirk et al., 2015).

Disquietingly, infectious causes of diarrhea reported in children in developing countries are common etiologies for diarrhea in young food-producing and companion animals in the United States (Cid et al., 2001; Kotloff et al., 2013; Martins et al., 2016; Moxley and Francis, 1986; Vidotto et al., 2013). It is estimated that 180 million kittens are born in the U.S. each year and inestimable numbers of these kittens are abandoned, orphaned, or relinquished to be fostered by thousands of U.S. animal shelters (Cave et al., 2002; Kitts-Morgan, 2015; New et al., 2004). While the exact statistics are unknown, approximately 15% of kittens fostered by these shelters will die or be euthanized because of severe illness before they reach 8-weeks of age (personal communications, Jim Babbitt, chief veterinarian San Diego Humane Society and SPCA and Mondy Lamb, marketing director Wake County SPCA). Infectious diseases are prevalent in this population and as many as 50% of kittens have diarrhea at the time of death or evidence of enteritis post-mortem (Cave et al., 2002;
Ghosh et al., 2013; Nutter et al., 2004; Sparkes et al., 2006). Very few studies have attempted to identify EPEC in the gastrointestinal tract or feces of cats (Goffaux et al., 2000; Krause et al., 2005; Mian, 1959; Morato et al., 2009; Pospischil et al., 1987; Puno-Sarmiento et al., 2013). Studies either evaluated very few cats or were performed on healthy animals only. These studies largely focused on animals as a reservoir for EPEC and not on an association of EPEC with diarrhea (Goffaux et al., 2000; Krause et al., 2005; Mian, 1959; Pospischil et al., 1987). Two previous studies focusing on adult cats identified a 1% prevalence of EPEC in cats with diarrhea and a slightly higher (3-6%) prevalence in healthy cats (Morato et al., 2009; Puno-Sarmiento et al., 2013).

In susceptible species, EPEC bacteria cause diarrhea and severe dehydration by attaching to the microvillus brush border of intestinal epithelial cells by means of the adhesin intimin, which is encoded by the enterocyte attaching and effacing (eae) gene (Jerse et al., 1990). During the course of investigating gastrointestinal lesions associated with death in kittens, we discovered a significant association between colonization of the intestinal epithelium by eae-positive E. coli and death or euthanasia due to severe illness (Ghosh et al., 2013). Given the established role of EPEC as a leading cause of diarrhea and diarrhea-related mortality in children we hypothesized a similar potential of EPEC infection in kittens. Consequently, the purpose of the present study was to determine the prevalence of EPEC in kittens ≤ 12 weeks of age and to establish any association between EPEC infection and diarrhea, diarrhea-related mortality, specific intestinal tract pathology, or factors promoting susceptibility to clinical disease. Our rationale was that identification of EPEC as an important cause of diarrhea and related mortality in kittens could provide a unique
opportunity for development of diagnostic, treatment, or prevention strategies having dual benefit to both kittens and children with EPEC infection.
Materials and Methods

Kitten case selection. Kittens that were ≤ 12 weeks of age, ≤ 1 kg body weight, unrelated, and housed separately were prospectively identified at two independent shelter facilities over a time period of 2 years. During phase I of the study, fecal samples were collected from live kittens with and without clinical signs of diarrhea. During phase II of the study, kittens that died or were euthanized due to severe clinical signs of diarrhea were selected. A cohort of apparently healthy kittens that were euthanized for non-health related reasons was selected as a control group. No kittens were euthanized for the purpose of the study. For all kittens, medical records were obtained when available. The study was approved by the North Carolina State University Institutional Animal Care and Use Committee.

Fecal sample collection and culture isolation of E. coli. Fecal samples were collected by shelter personnel (phase I) or were collected from each kitten at the time of autopsy by study investigators (phase II). Feces (live kittens) and rectal contents (kittens that died) were additionally swabbed by shelter personnel for preservation of fastidious bacteria. Samples and Cary Blair swabs (Beckton, Dickinson and Company, Franklin Lakes, NJ) were transported to the laboratory on ice packs within 24 hours of collection. Culture swabs were streaked for isolation onto MacConkey agar and incubated at 37°C overnight for detection of enteric, gram-negative bacteria. For each sample, 12 morphologically distinct, lactose-positive bacterial colonies were sub-cultured onto blood agar plates. Subcultures identified as indole positive, PYR negative, and oxidase negative by dryslides (Beckton, Dickinson and Company, Franklin Lakes, NJ) were determined to be E. coli. Isolates of E. coli were frozen in lysogeny or Luria-Bertani broth (LB)-glycerol at -80°C.
Identification of EPEC cultured from live kittens. Isolates of *E. coli* were heated at 100°C for 30 minutes for DNA extraction. Conventional PCR was performed to identify presence of the intimin gene (*eae*). If positive for *eae*, the isolate was additionally tested for the presence of genes encoding bundle forming pilus (*bfp*), and Shiga toxins 1 and 2 (*stx1, stx2*). All conventional PCR analyses were performed using published primer sequences and reaction conditions and AmpliTaqGold DNA polymerase (Thermo Fisher Scientific, Waltham, MA) (Franck et al., 1998; Gunzburg et al., 1995; Jerse et al., 1990; Kobayashi et al., 2001; Paton et al., 1993). All EPEC isolates were further confirmed as *E. coli* by use of the Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) analyzer Vitek® MS (Biomérieux, Marcy l’Etoile, France) as previously described for gram-negative bacteria (Stephan et al., 2011).

Multiplex PCR for *E. coli* virulence gene expression. Individual isolates of *E. coli* obtained during phase II of the study were shipped overnight on LB agar for testing by means of multiplex PCR (*E. coli* Reference Center, Pennsylvania State University, University Park, PA) for the presence of virulence genes encoding intimin (*eae*), Shiga toxins 1 and 2 (*stx1, stx2*), heat-stable and heat-labile toxins (*STa, STb, LT*), invasion plasmid antigen H (*ipaH*), enteroaggregative gene (*Eagg*), and cytotoxic necrotizing factors 1 and 2 (*cnf1, cnf2*) using published protocols (DebRoy and Maddox, 2001). Conventional PCR for identification of *bfp* was performed as previously described (Gunzburg et al., 1995).

Fecal DNA extraction. DNA was extracted from 100 mg fecal samples using a commercial kit (Zymo Research, Irvine, CA) as previously described and stored at -80°C (Stauffer et al., 2008).
**Quantitative PCR.** Quantitative PCR was used to amplify bacterial 16S and *E. coli* 16S rRNA from extracts of fecal DNA. Primers used for bacterial 16S were previously published (Ritchie et al., 2008). Primers used for *E. coli* 16S were F-5’-CATGCCGCGTGTATGAAGAA-3’ and R-5’-CGGGTAACGTCAATGAGCAAA-3’.

Primers were used at a concentration of 0.5 µM in a 20 µl reaction volume with commercially available qPCR master mix (PerfeCTa SYBR supermix for iQ, Quantabio, Beverly, MA). Amplifications were performed with an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60.7°C for 45 seconds, and extension at 68°C for 1 minute.

A quantitative PCR assay for *eae* was optimized and validated for quantifying EPEC in feces. An isolate of EPEC from a kitten with diarrhea was inoculated in LB, incubated overnight at 37°C, and serially diluted ten-fold in sterile PBS (10^1 to 10^8). The dilutions were simultaneously plated for CFU counts and spiked into 100 mg aliquots of feline feces prior to DNA extraction. Primers used for amplification of *eae* were previously published (Franck et al., 1998; Yu and Kaper, 1992). Amplifications were performed with an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 45 seconds, and extension at 68°C for 1 minute.

**Autopsy and sample collection.** Deceased kittens were kept at 4°C for less than 24 hours and then transported on ice from each collaborating shelter to the laboratory for autopsy. The gastrointestinal tract from distal esophagus to distal colon was removed and opened longitudinally to expose the entire lumen. The gastrointestinal tract and colonic contents of each kitten were photographed. Fecal samples were obtained from the colon for culture.
isolation of *E. coli* and DNA extraction. The gastrointestinal tract was then immersed in phosphate buffered saline and shaken to dislodge contents. Grossly abnormal section(s) of the gastrointestinal tract were described and selectively marked for later inclusion during tissue sampling. The entire gastrointestinal tract was then submerged in 10% neutral buffered formalin for a minimum of 24-48 hours. The gastrointestinal contents and remaining feces were stored at 4°C until utilized for parasitological examination.

**Fecal scoring.** Fecal scores were assigned by 3 individuals blinded to kitten health status, utilizing photographs of colonic content that were taken at the time of autopsy. Fecal score was calculated based on a published scale ranging from 1 (very hard and dry) to 7 (watery with no texture) (Bybee et al., 2011). A median fecal score was calculated from the score of each individual. If the colon was empty, perineal soiling was considered representative of severe diarrhea and a score of 7. No score was assigned if the colon was empty and no perineal soiling was present.

**Histopathology.** Full-thickness samples of the stomach (fundus and antrum), proximal duodenum, mid-jejunum, ileum, and colon (proximal and distal) were obtained from the formalin-fixed gastrointestinal tract of each kitten. Any grossly abnormal tissue that was identified at autopsy was also included and marked with India ink. The sampled tissues were paraffin embedded, sectioned at thickness of 5 µm and stained with hematoxylin and eosin or Giemsa. Microscopic examination of each tissue was performed by an American College of Veterinary Pathologists boarded pathologist (V.E.W.) that was blinded to the identity of each kitten. Hematoxylin and eosin stained sections from each gastrointestinal region were examined and scored based on a simplified histopathological model for determining
gastrointestinal inflammation (Jergens et al., 2014). Each lesion was scored as absent (0), mild (1), moderate (2), or severe (3). In addition to scoring inflammation, sections were evaluated for lesions consistent with or indicative of specific gastrointestinal disease or etiology. The severity of autolysis was recorded using the following guidelines: lifting of epithelial cells from the villi (mild); sloughed epithelial cells (moderate); and hypereosinophilia with loss of tissue architecture (severe). Giemsa stained sections were specifically evaluated for the presence of bacteria in close association with the brush border of the intestinal epithelium.

**Fluorescence in situ hybridization.** Formalin-fixed and paraffin-embedded 5 µm sections of gastrointestinal tissue from each kitten were mounted on poly-L-lysine-coated slides and fluorescence in situ hybridization (FISH) was performed as previously described (Ghosh et al., 2013; Janeczko et al., 2008; Nicklas et al., 2010; Simpson et al., 2006). Probes used for hybridizations included a universal eubacterial probe Eub338 (5’-GCTGCCTCCCGTAGGAGT-6-FAM-3’) (Janeczko et al., 2008), a probe specific for *E. coli/Shigella* (5’-Cy3-GCAAAGGTATTAACTTTACTCC-3’) (Janeczko et al., 2008), and a negative control non-Eub probe (5’-Cy3-CGACGGAGGCQTCCTCA-3’). Probes were reconstituted with sterile water and diluted with hybridization buffer to final working concentrations of 5 ng/µl. Formalin-fixed and paraffin-embedded intestinal tissue from a puppy diagnosed with enteroadherent *E. coli* was included in each hybridization experiment as a positive control.

**Parasitological examination.** Gastrointestinal contents were grossly examined for the presence of helminths using a dissecting microscope. Collected helminths were fixed and
processed as previously described (Pritchard, 1982). Three parasitological techniques were utilized for analysis of each fecal sample. For direct fecal microscopic examination, a scant amount of feces (micrograms) was mixed in 0.9% saline on a microscope slide and covered with a 22 mm square cover slip. The sample was systematically examined at 100x magnification with a compound microscope for the detection of helminth ova and larvae and again at 400x magnification for protozoan cysts and trophozoites. Fecal centrifugation was used for detection of coccidian oocysts and nematode and cestode ova as previously detailed (Dryden et al., 2005). Fecal material (≤ 1 gram) was mixed in sodium nitrate flotation solution at a specific gravity of 1.20, strained, and centrifuged for 5 minutes. After centrifugation, additional flotation solution was added to the tube mixture until a meniscus formed. A 22 mm square cover slip was added to the meniscus and allowed to stand for 10 minutes. The cover slip was transferred to a microscope slide and the sample was systematically examined for helminth ova at 100x magnification. Fecal sedimentation was performed for detection of trematode eggs and nematode larvae. Fecal material (≤ 1 gram) was mixed in 0.9% saline, strained, and centrifuged for 5 minutes. After centrifugation, sediment from the bottom of the centrifuge tube was transferred with a Pasteur pipette to a microscope slide. The sample was covered with a 22 x 40 mm cover slip and systematically examined for helminth ova and nematode larvae at 100x magnification. The transfer and examination of sediment from the bottom of the centrifuge tube was repeated until all the sediment was examined.

**Medical record review.** If available, individual medical records were reviewed. Recorded parameters included: age, sex, weaning status, presence and nature of ante-mortem clinical
signs, medications and vaccinations administered, whether euthanized or died, time in foster care, source (shelter), and body weight.

**Statistical analysis.** Discrete data were analyzed for significant differences in observations between groups (number of kittens) by $\chi^2$ test and odds ratio or Fisher exact test. Continuous data were analyzed for significant differences in mean and/or median values between groups using Student’s t test and Mann-Whitney rank sum test, respectively. Continuous data was graphed as vertical point plots and whisker-box plots using the Tukey method. The linear correlation between variables was determined using the linear regression line of plotted data as well as Pearson product moment correlation tests. Statistical analyses were performed using commercial software (SigmaPlot 12, Systat Software, Inc., San Jose, CA) and significance was assigned a P value of < 0.05.
Results

Naturally-occurring atypical EPEC infection is prevalent in kittens. Feces from 61 live kittens from 2 different shelter facilities in North Carolina were evaluated for the presence of EPEC (Table 1). *Escherichia coli* was cultured from the feces of all but one kitten. Among the 60 kittens with *E. coli* isolated, positive cultures for EPEC (*eae*, *stx1*-, and *stx2*-) were obtained from 11 kittens, resulting in an overall prevalence of infection of 18%. There was no significant difference in the culture of EPEC from feces of kittens with (6/27; 22%) compared to those without diarrhea (5/33; 15%). All EPEC isolates were identified as atypical based on absence of the gene coding for bundle forming pilus (*bfp*) (Gunzburg et al., 1995).

Compared to results of non-selective fecal culture for isolation of EPEC, the gene for intimin (*eae*) could be directly amplified by means of qPCR from the fecal DNA of 22 kittens, suggesting a prevalence of EPEC exposure or infection as high as 36%. There was no significant difference in prevalence of *eae* in feces from kittens with (13/28; 46%) compared to those without diarrhea (9/33; 27%) (Figure 1 B). Based on analysis of qPCR for *eae*, fecal DNA of kittens with diarrhea had a numerically greater quantity of *eae* present compared to kittens without diarrhea (Figure 1 C); however, this association was not statistically significant. Based on a standard curve correlation of *eae* cycle threshold (*C*<sub>t</sub>) to colony forming units (CFU) of EPEC in feces (Figure 1 A), kittens with diarrhea were estimated to shed a median of $4.9 \times 10^5$ CFU (IQR = $2.5 \times 10^4$ – $4.7 \times 10^6$) of EPEC per 100 mg feces compared to $2.8 \times 10^4$ CFU (IQR = $3.8 \times 10^3$ – $1.5 \times 10^5$) of EPEC by kittens without diarrhea. Quantitative PCR for amplification of 16S rDNA from feces was not different.
between the kittens. Fecal DNA from each kitten failed to amplify the presence of genes for
\textit{stx1}, \textit{stx2}, or \textit{hfp}.

\textbf{Diarrheal mortality is associated with a greater quantity of EPEC.} Recent epidemiologic
studies in children have identified that EPEC infection is associated with a higher hazard of
diarrheal death (Kirk et al., 2015; Kotloff et al., 2013). To determine if there is any
association between EPEC infection and diarrheal mortality in kittens, we first identified the
pathotypes of \textit{E. coli} that could be cultured from the feces of apparently healthy kittens
compared to kittens that died or were euthanized due to severe diarrhea. Fifty-four deceased
kittens from two different shelter facilities had feces cultured for the presence of
diarrheagenic \textit{E. coli} including EPEC (Table 2). Apparently healthy kittens all came from a
single shelter and were euthanized after short-term housing because they were feral or
otherwise unadoptable. In contrast, kittens with diarrhea were predominantly from a different
shelter in which foster care was provided until the time of their death or euthanasia due to
severe diarrhea. Kittens with diarrhea had a significantly lower body weight than apparently
healthy kittens and a significantly longer time period lapse between death and autopsy. A
diarrheagenic pathotype of \textit{E. coli} was cultured from the feces of 9/19 (47\%) apparently
healthy kittens and 10/29 (34\%) kittens that died or were euthanized due to diarrhea (Table
3). The most common pathotypes of \textit{E. coli} identified were EPEC and necrotoxigenic \textit{E. coli}
(NTEC). Enteropathogenic \textit{E. coli} was cultured from feces of 3/19 (16\%) apparently healthy
kittens and 6/29 (21\%) kittens dying with diarrhea. In contrast, \textit{eae} could be directly
amplified from the fecal DNA of 8/20 (40\%) apparently healthy kittens and 18/34 (53\%)
kittens dying with diarrhea (Figure 2 A). There was no significant difference in the culture or
qPCR-based prevalence of EPEC infection between apparently healthy kittens and kittens that died or were euthanized due to diarrhea. However, all of the E. coli culture isolates from kittens with EPEC that died or were euthanized due to diarrhea were identified as EPEC. Furthermore, kittens with eae that died or were euthanized due to diarrhea had a significantly greater quantity of eae in their feces compared to apparently healthy kittens with amplification of eae (Figure 2 B). Based on quantity of eae determined by qPCR, kittens dying with diarrhea and concurrent eae amplification shed an estimated median of $1.8 \times 10^6$ CFU (IQR = $2.3 \times 10^5 – 5.3 \times 10^7$) of EPEC per 100 mg feces compared to $1.4 \times 10^4$ CFU (IQR = $7.7 \times 10^3 – 3.1 \times 10^6$) of EPEC by kittens without diarrhea. Quantitative PCR for amplification of 16S rDNA from feces was not different between the two groups of kittens. Fecal DNA from each kitten failed to amplify the presence of genes for stx1, stx2, or bfp.

**Gross and microscopic lesions are common in the small intestine and colon of kittens with severe diarrhea.** Based on the photographic appearance of colonic contents at autopsy, kittens that died or were euthanized due to diarrhea were confirmed to have significantly higher fecal scores as compared to apparently healthy kittens (Table 4). Four kittens with diarrhea (n=34) and one apparently healthy kitten (n=20) did not have feces scored due to absence of colonic content or perineal fecal soiling. Gross lesions were observed at the time of autopsy in 32/34 (94%) kittens that died or were euthanized due to diarrhea and in only 7/20 (35%) apparently healthy kittens (Table 4). In kittens with diarrhea, gross and light microscopic lesions were significantly more common and severe in the small intestine and colon. In the small intestine, diarrhea was significantly associated with crypt dilation or distortion. In the colon, diarrhea was significantly associated with epithelial injury. Among
all kittens undergoing autopsy, there was no association between India-inked gross lesions and any specific lesion identified on light microscopic examination of the corresponding tissue section (Supplementary Table 1 and Supplementary Figure 1). No gross lesions were identified to be specifically associated with the presence of EPEC (Table 4).

**Kitten EPEC infection is associated with greater severity of inflammatory infiltrates in the small intestine and colon.** Based on the photographic appearance of colon contents at the time of autopsy, kittens with evidence of EPEC infection (i.e. *eae*-positive and/or EPEC cultured) did not have more severe diarrhea than kittens in which evidence of EPEC infection was not identified (Table 4). However, kittens with diarrhea and positive culture or *eae* qPCR results for EPEC had significantly greater small intestinal and colonic lesion scores compared to healthy kittens and kittens with diarrhea but no evidence of EPEC as determined by culture and qPCR (Table 4 and Figure 3). The increase in small intestinal lesion scores in kittens with diarrhea and EPEC was due to increased epithelial injury and inflammatory infiltrate in the lamina propria. The increase in colonic lesion scores in kittens with diarrhea and EPEC was due to increased inflammatory infiltrate in the lamina propria (Table 4 and Figure 4). Autolysis was observed in intestinal tissue sections from 33 of the 54 kittens (61%) and was significantly more common in apparently healthy kittens (Table 4).

**Enteroadherent *E. coli* was demonstrated in the intestines of kittens that died or were euthanized due to diarrhea.** Light microscopic examination of Giemsa stained sections of gastrointestinal tissue obtained from each kitten identified the presence of mucosa-associated bacteria and/or debris in 17/34 (50%) kittens that were euthanized or died due to severe diarrhea and 5/20 (25%) apparently healthy kittens. Using an *E. coli*-specific oligonucleotide
probe to identify the presence of mucosa-associated \textit{E. coli} by means of fluorescence \textit{in situ} hybridization, enteroadherent \textit{E. coli} was observed in the small intestine and colon, respectively, of two kittens that were euthanized due to diarrhea (Figure 5). One kitten was both qPCR positive for \textit{eae} and EPEC was cultured, while the other kitten was qPCR positive for \textit{eae} only. Other mucosa-associated bacteria that did not fluoresce with the \textit{E. coli} specific probe were identified in 6 kittens with diarrhea (spirochetes and rods) and 4 apparently healthy kittens (segmented filamentous bacteria and rods).

\textbf{There was no association between EPEC infection and intestinal parasitism.}

Gastrointestinal parasites were identified in 28/51 (55\%) autopsied kittens. Three kittens that died or were euthanized due to diarrhea did not contain enough feces to perform parasite evaluations. Intestinal parasites were more often identified in the apparently healthy kittens compared to kittens that were euthanized or died due to diarrhea (Table 5 and Supplementary Figure 2). This difference could be attributed to infrequent administration of preventative medications to euthanized healthy kittens as documented in their medical records (Supplementary Table 2). Parasites identified in kittens that were culture or \textit{eae} qPCR positive for EPEC infection included \textit{Toxocara cati} in 10/27 (37\%) cats and \textit{Isospora} spp. in 8/27 (30\%) cats. There was not a statistically significant association between EPEC infection and the presence of intestinal parasites.

\textbf{EPEC infection was significantly associated with need for administration of parenteral fluids.} To determine if any individual, environmental, clinical, or therapeutic variables were associated with increased odds of EPEC infection, all objective data that could be obtained from the medical record of each kitten was examined. A summary of these variables and their
association with combined results of EPEC culture and eae qPCR for each group of kittens is shown in Table 6. Live kittens with EPEC were significantly more likely to have a decreased appetite and to have received parenteral fluids. When all live and deceased kittens were combined into groups based on EPEC culture and eae qPCR, EPEC was significantly associated with need for parenteral fluid administration (OR 3.36, 95% CI 1.34 – 8.41, P=0.015). The medications given to kittens within each category in Table 6 are shown in supplementary Table 2.
Discussion

This study identified that 40% of kittens sampled post-mortem from two different shelter environments in the United States shed isolates of diarrheagenic *Escherichia coli* (DEC) in their feces. Most of the DEC isolated from these kittens were identified as atypical enteropathogenic *E. coli* (aEPEC) or necrotoxigenic *E. coli* based on virulence pathotyping. We chose to focus on investigation of EPEC in this population based on our prior findings of EPEC colonizing the intestinal epithelium of kittens that died while under foster care (Ghosh et al., 2013) and recent results of a large case-controlled study conducted in sub-Saharan Africa where EPEC was associated with a significantly higher hazard of death in children with moderate to severe diarrhea (Kotloff et al., 2013). We hypothesized that EPEC may be similarly associated with the high diarrheal mortality reported in kittens in the United States.

Based on positive results of fecal culture, EPEC infection was identified in 18% of kittens in this study. This prevalence is higher than reported in previously published studies of EPEC infection in cats (Krause et al., 2005; Morato et al., 2009; Puno-Sarmiento et al., 2013) and is likely due to our focus on kittens. In developing countries, the prevalence of EPEC infection in children with diarrhea is 8% and the hazard of diarrheal death in children with EPEC is the greatest for infants under the age of 11 months (Dias et al., 2016; Kotloff et al., 2013; Ochoa et al., 2011). In this study, EPEC were cultured from feces of apparently healthy kittens, live kittens with diarrhea, and kittens that died or were euthanized due to severe clinical signs of diarrhea. This is similar to reports of EPEC in children where the prevalence of infection is similar in individuals with diarrhea compared to those without diarrhea.
In this study, a non-significantly higher percentage of kittens with diarrhea were culture positive for EPEC compared to kittens without diarrhea. Additionally, 100% of *E. coli* isolates cultured from EPEC-positive kittens dying with diarrhea were identified as EPEC. Therefore, to investigate whether diarrhea was related to the burden of EPEC infection, we developed a qPCR assay for quantification of *eae* in DNA extracted from feces of kittens. As a “surrogate” for detection of EPEC, *eae* was amplified from the feces of 42% of kittens in the study; a much larger percentage than was identified by fecal culture for EPEC. While the percentage of kittens that were positive for *eae* did not differ significantly between apparently healthy kittens, live kittens with diarrhea, and kittens that died or were euthanized due to severe clinical signs of diarrhea, there was a significant association between increased quantity of *eae* in feces and diarrheal death. These findings indirectly suggest that higher quantities of EPEC are significantly associated with diarrhea-related mortality in kittens. These results are similar to studies of children where EPEC load, as measured by qPCR, was higher in individuals with diarrhea as compared to those with asymptomatic EPEC infection (Barletta et al., 2011). It is tempting to propose utilization of qPCR for *eae* as an alternative to the more laborious fecal culture techniques otherwise needed to diagnose EPEC infection. This approach has been applied to insightful epidemiological studies of EPEC infection in children (Barletta et al., 2011; Liu et al., 2016). Unfortunately, a high prevalence of *eae*-positivity in kittens and children with and without diarrhea precludes the use of qPCR for determination of disease causation. This is particularly true in kittens and children with clinical disease in which a myriad of potential infectious agents may be responsible for diarrhea and positive results of qPCR cannot
differentiate live versus dead bacteria. The potential for horizontal gene transfer of eae between different bacteria make the results of eae qPCR incompletely specific for EPEC (Deng et al., 2001). Nonetheless, based on our positive EPEC culture results, it is likely that positive qPCR amplification of eae demonstrated kittens with a recent history of EPEC infection. More importantly, this study demonstrated that an increased quantity of eae in feces is significantly associated with diarrheal mortality in kittens.

Very few studies have described the pathological lesions associated with EPEC infection in children (Fagundes-Neto et al., 1997; Hill et al., 1991; Rothbaum et al., 1982; Ulshen and Rollo, 1980). The main histopathological findings in children with EPEC are villus atrophy, crypt hyperplasia, enteroadherent bacteria, and mucosal inflammatory infiltrate in the small intestine (Fagundes-Neto et al., 1997; Hill et al., 1991; Rothbaum et al., 1982; Ulshen and Rollo, 1980). In this study, we identified a significant association between EPEC and lesions in both the small intestine and colon of kittens that died or were euthanized due to diarrhea. Lesions were defined by the presence of epithelial injury in the small intestine and an inflammatory infiltrate in the small intestine and colon. The median inflammation score in the small intestine was higher than that in the colon, suggesting that the pathological effects of EPEC in kittens may be more pronounced in the small intestine, similar to EPEC infection in children (Hill et al., 1991; Rothbaum et al., 1982; Ulshen and Rollo, 1980). Both epithelial injury and inflammatory cell infiltrate could contribute directly to pathogenesis of EPEC diarrhea by mechanisms related to increased paracellular permeability, intestinal malabsorption, and increased epithelial secretion. Cell culture-based studies of EPEC pathogenesis support the presence of effector proteins that can alter
permeability of the tight junction barrier, electrolyte transport, apical plasma membrane microvillus architecture and function, and promote secretion of pro-inflammatory cytokines (Dean and Kenny, 2004; Gill et al., 2007; Kenny et al., 1997; McNamara et al., 2001; Raymond et al., 2011; Shaw et al., 2005; Thanabalasuriar et al., 2010; Tomson et al., 2005). While the observed histopathological lesions were significantly more severe in kittens with EPEC infection, they lack specificity and were also observed in kittens with diarrhea that did not have EPEC.

Based on results of FISH utilizing a DNA probe specific for *E. coli*, colonization of both the small intestine and colon was evidenced by palisades of *E. coli* attached to either the villus or crypt epithelium. We observed a lower prevalence of FISH-positive results for EPEC in this study (2/54; 3.7%) compared to our prior study (9/50; 18%) in which kittens with all causes of mortality were examined (Ghosh et al., 2013). The reason for this difference is unclear but may be due to differences in medications (such as antibiotics) administered to kittens with diarrhea or a higher prevalence of autolysis of tissues from kittens in the present study. Nonetheless, the number of FISH positive kittens is likely a gross underestimate based on the size of the sample examined compared on scale to the entire gastrointestinal tract.

Despite a large number of insightful studies of EPEC pathogenesis using *in vitro* models of the infection, a significant gap in knowledge exists regarding what host or bacterial factors are responsible for why some EPEC infected children remain asymptomatic while others develop severe diarrhea that can result in death. Examination of the demographic, environmental, clinical, and treatment history of individual kittens from both
live and deceased populations failed to disclose any factors distinguishing between EPEC infected kittens with diarrhea versus without diarrhea. We hypothesized that weaning would be associated with increased susceptibility to EPEC diarrhea due to an association with stress, altered intestinal function, and increased susceptibility to infection and disease in multiple species (Moeser et al., 2007; Rakoff-Nahoum et al., 2015). In addition, maternal milk has been shown to protect against EPEC infection in rabbits and infants (Blake et al., 1993; Gallois et al., 2007; Rogier et al., 2014). In the present study, nearly all of the kittens were weaned, which likely precluded our ability to meaningfully assess the impact of weaning on susceptibility to EPEC diarrhea.

Given the myriad medications administered to kittens in this study for the treatment of many ostensible causes for diarrhea, it is remarkable that kittens diagnosed with EPEC infection were significantly more likely than other kittens to have received parenteral fluid administration. This finding implies that kittens with EPEC infection were likely to have a greater severity of dehydration compared to kittens with other causes of diarrhea in the study. Greater severity of dehydration in kittens with EPEC parallels the association of EPEC with increased severity of intestinal histopathological lesions and suggests greater fecal water loss in kittens with EPEC diarrhea. It is worth considering that greater dehydration in kittens and children with EPEC infection may contribute to the increased hazard of death associated with EPEC infection.

Several limitations to this study are worth mentioning. First, the study was undertaken in kittens under naturally-occurring conditions in two different shelter facilities with different population sizes, processing procedures, and foster-care policies. While adding considerable
extraneous variability to the study, we did not find any statistically significant differences in EPEC infection in kittens based on shelter identity. Secondly, in both live and deceased kitten populations, apparently healthy kittens spent a significantly shorter time in foster care and received fewer medications and preventatives compared to kittens with diarrhea. In healthy kittens, fecal samples were mostly obtained within 24 hours of shelter intake, prior to administration of preventatives, and then kittens were transferred to foster care (live kittens) or euthanized due to feral nature, overpopulation, or arising health concerns in littermates (deceased kittens). In contrast, kittens with diarrhea were returned dead or alive to the shelter by their foster caregiver for purposes of this study or for clinical examination by the shelter veterinarian respectively. Despite this limitation, we observed no significant association between days in foster care and the presence of EPEC infection in this study. Finally, with the exception of parasitological and histopathological examination, we did not conduct a diagnostic investigation for other infectious culprits of diarrhea in kittens with or without EPEC. Accordingly, results of our study do not identify EPEC as a definitive cause for diarrhea or diarrheal death in the kittens reported here.

Overall, this study demonstrates a strong association between burden of EPEC infection, as determined by eae qPCR, and the presence of histopathological lesions of epithelial injury and inflammatory infiltrate in the intestinal tract, increased need for parenteral fluid administration, and diarrhea-related mortality in shelter kittens. EPEC infection in kittens mirrors the prevalence, population demographics, circumstances, and clinical observations of EPEC infection in children. Accordingly, studies of the infection in kittens with and without diarrhea offer a unique opportunity to investigate host and bacterial
factors responsible for mediating disease susceptibility and may lead to novel treatment approaches to ameliorate EPEC-associated diarrhea in kittens and children.
Tables

Table 1. Demographic data from 61 live kittens with and without clinical signs of diarrhea.

<table>
<thead>
<tr>
<th>Demographic Variable</th>
<th>No Diarrhea (n=33)</th>
<th>Diarrhea (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) kittens source 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 (73)</td>
<td>23 (82)</td>
</tr>
<tr>
<td>No. (%) kittens source 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 (27)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Age in weeks</td>
<td>6.3 ± 1.8</td>
<td>7.0 ± 2.3</td>
</tr>
<tr>
<td>Sex</td>
<td>11 males, 21 females, 1 unknown</td>
<td>14 males, 13 females, 1 unknown</td>
</tr>
<tr>
<td>Weight (range) in grams</td>
<td>480 (280 – 909)</td>
<td>682 (227 – 1000)</td>
</tr>
<tr>
<td>No. (%) weaned</td>
<td>33 (100)</td>
<td>26 (93)</td>
</tr>
<tr>
<td>Days (range) in foster care</td>
<td>0 (1 – 30)</td>
<td>9 (0 – 42) ***</td>
</tr>
<tr>
<td>No. (%) concurrent evidence of URD</td>
<td>3 (9)</td>
<td>6 (21)</td>
</tr>
<tr>
<td>No. (%) decreased appetite</td>
<td>1 (3)</td>
<td>2 (7)</td>
</tr>
</tbody>
</table>

Data represent number (No) and percent (%) of kittens, mean ± SD for age, or median (range) for weight and days in foster care. URD: Upper respiratory tract disease

<sup>a</sup> County Animal Center

<sup>b</sup> County SPCA

*** P < 0.001; Mann-Whitney Rank Sum test
Table 2. Demographic data from 54 deceased kittens that were apparently healthy or had died or were euthanized due to diarrhea.

<table>
<thead>
<tr>
<th>Demographic Variable</th>
<th>No Diarrhea (n=20)</th>
<th>Diarrhea-associated mortality (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) kittens source 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 (100)</td>
<td>8 (24)</td>
</tr>
<tr>
<td>No. (%) kittens source 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>26 (76)</td>
</tr>
<tr>
<td>Age in weeks</td>
<td>6.5 ± 2.5</td>
<td>5.6 ± 1.8</td>
</tr>
<tr>
<td>Sex</td>
<td>11 males</td>
<td>16 males</td>
</tr>
<tr>
<td></td>
<td>9 females</td>
<td>18 females</td>
</tr>
<tr>
<td>Weight (range) in grams</td>
<td>538 (261 – 896)</td>
<td>326 (163 – 598)**</td>
</tr>
<tr>
<td>No. (%) weaned</td>
<td>15 (75)</td>
<td>27 (79)</td>
</tr>
<tr>
<td>Days (range) in foster care</td>
<td>3.0 (0 – 11)</td>
<td>8.5 (0 – 37)**</td>
</tr>
<tr>
<td>No. (%) concurrent evidence of URD</td>
<td>3 (15)</td>
<td>7 (21)</td>
</tr>
<tr>
<td>No. (%) decreased appetite</td>
<td>0 (0)</td>
<td>24 (71)**</td>
</tr>
<tr>
<td>Time (range) in hours from death to necropsy</td>
<td>3.0 (1 – 24)</td>
<td>6.75 (1 – 24)**</td>
</tr>
</tbody>
</table>

Data represent number (No.) and percent (%) of kittens, mean ± SD, or median (range).
URD: Upper respiratory tract disease
<sup>a</sup> County Animal Center
<sup>b</sup> County SPCA
**P<0.01, ***P<0.001; Mann-Whitney Rank Sum test
***P<0.001 Χ² (appetite)
Table 3. Results of multiplex PCR for detection of virulence gene(s) in *E. coli* cultured from feces of apparently healthy kittens and kittens that died or were euthanized due to diarrhea.

<table>
<thead>
<tr>
<th>Culture Finding</th>
<th>No Diarrhea (n=20)</th>
<th>Diarrhea-associated mortality (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%), Kittens</td>
<td>No. (%), Isolates</td>
<td>No. (%), Hemolytic isolates</td>
</tr>
<tr>
<td>Positive fecal bacterial culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation of <em>E. coli</em></td>
<td>19/20 (95%)</td>
<td></td>
</tr>
<tr>
<td><em>Diarrheagenic E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enteropathogenic (<em>eae</em>+/<em>stx</em>-*)</td>
<td>3/19 (16%)</td>
<td>6</td>
</tr>
<tr>
<td>Necrotoxigenic (<em>cnf1</em>+)</td>
<td>5/19 (26%)</td>
<td>10</td>
</tr>
<tr>
<td>Enterotoxigenic (<em>STa</em>+/<em>STb</em>+)</td>
<td>1/19 (5%)</td>
<td>3</td>
</tr>
<tr>
<td>Enterohemorrhagic (<em>eae</em>+/<em>stx</em>+)</td>
<td>0/19 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>Enteroaggregative (<em>Eagg</em>+)</td>
<td>0/19 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>Enteroinvasive (<em>ipaH</em>)</td>
<td>0/19 (0%)</td>
<td>0</td>
</tr>
<tr>
<td><em>Non-diarrheagenic E. coli</em></td>
<td>8/19 (42%)</td>
<td>30/49 (61%)</td>
</tr>
</tbody>
</table>
cnf1: cytotoxic necrotizing factor 1, STa/STb: heat stable toxins a/b, eae: enterocyte attaching and effacing, stx: shiga-like toxin, Eagg: enteroaggregative gene, ipaH: invasion plasmid antigen H

a One kitten had *E. coli* with both *cnf1* and *eae*
b One kitten had *E. coli* with both *cnf1* and *Eagg*
Table 4. Gross and light microscopic lesion scores obtained from apparently healthy kittens and kittens that were euthanized or died due to severe diarrhea and on the basis of EPEC culture and/or qPCR for eae.

<table>
<thead>
<tr>
<th>Kitten categories</th>
<th>No diarrhea (n=20)</th>
<th>Diarrhea-associated mortality (n=34)</th>
<th>No diarrhea (n=11)</th>
<th>EPEC + (n=9)</th>
<th>EPEC – (n=16)</th>
<th>EPEC + (n=18)</th>
<th>EPEC – (n=27)</th>
<th>EPEC + (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fecal score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No diarrhea</td>
<td>4 (2 – 6)</td>
<td>7 (2 – 7)**</td>
<td>5 (3.5 – 6)</td>
<td>4 (2 – 6)</td>
<td>7 (4 – 7)</td>
<td>7 (2 – 7)</td>
<td>6 (3.5 – 7)</td>
<td>6 (2 – 7)</td>
</tr>
<tr>
<td><strong>Gross lesions (No.)</strong></td>
<td>7/20 (35)</td>
<td>32/34 (94)**</td>
<td>3/11 (27)</td>
<td>4/9 (44)</td>
<td>16/16 (100)</td>
<td>16/18 (89)</td>
<td>19/27 (70)</td>
<td>20/27 (74)</td>
</tr>
<tr>
<td>Stomach</td>
<td>1/20 (5)</td>
<td>6/34 (1)</td>
<td>1/11 (9)</td>
<td>0/9 (0)</td>
<td>3/16 (19)</td>
<td>3/18 (17)</td>
<td>4/27 (15)</td>
<td>3/27 (11)</td>
</tr>
<tr>
<td>Small intestine</td>
<td>7/20 (35)</td>
<td>25/34 (74)*</td>
<td>3/11 (27)</td>
<td>4/9 (44)</td>
<td>12/16 (75)</td>
<td>13/18 (72)</td>
<td>15/27 (56)</td>
<td>17/27 (63)</td>
</tr>
<tr>
<td>Colon</td>
<td>0/20 (0)</td>
<td>9/34 (26)*</td>
<td>0/11 (0)</td>
<td>0/9 (0)</td>
<td>4/16 (25)</td>
<td>5/18 (28)</td>
<td>4/27 (15)</td>
<td>5/27 (19)</td>
</tr>
<tr>
<td>Perineum</td>
<td>0/20 (0)</td>
<td>11/34 (32)**</td>
<td>0/11 (0)</td>
<td>0/9 (0)</td>
<td>7/16 (44)</td>
<td>4/18 (22)</td>
<td>8/27 (30)</td>
<td>5/27 (19)</td>
</tr>
<tr>
<td>Other</td>
<td>0/20 (0)</td>
<td>2/34 (6)</td>
<td>0/11 (0)</td>
<td>0/9 (0)</td>
<td>1/16 (6)</td>
<td>1/18 (6)</td>
<td>1/27 (4)</td>
<td>1/27 (4)</td>
</tr>
<tr>
<td><strong>Microscopic lesion score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus atrophy/stunting</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 2)</td>
<td>1 (0 – 2)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 2)</td>
</tr>
<tr>
<td>Epithelial injury</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 3)</td>
<td>0 (0 – 2)</td>
<td>0.5 (0 – 2)</td>
<td>0 (0 – 2)</td>
<td>1 (0 – 3)*</td>
<td>0 (0 – 2)</td>
<td>1 (0 – 3)*</td>
</tr>
<tr>
<td>Crypt dilation/ distortion</td>
<td>0 (0 – 1)</td>
<td>1 (0 – 3)**</td>
<td>0 (NR)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 3)</td>
<td>1 (0 – 3)</td>
<td>0 (0 – 3)</td>
<td>0 (0 – 3)</td>
</tr>
<tr>
<td>IEL</td>
<td>0 (0 – 2)</td>
<td>1 (0 – 3)</td>
<td>0 (0 – 2)</td>
<td>0.5 (0 – 2)</td>
<td>1 (0 – 3)</td>
<td>1 (0 – 2)</td>
<td>0.5 (0 – 3)</td>
<td>1 (0 – 2)</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>1 (0 – 3)</td>
<td>2 (0 – 3)</td>
<td>1 (0 – 2)</td>
<td>2 (0 – 3)*</td>
<td>1 (0 – 3)</td>
<td>2 (0 – 3)*</td>
<td>1 (0 – 3)</td>
<td>2 (0 – 3)*</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial injury</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 3)*</td>
<td>0 (0 – 1)</td>
<td>0 (NR)</td>
<td>0 (0 – 3)</td>
<td>0 (0 – 3)</td>
<td>0 (0 – 3)</td>
<td>0 (0 – 3)</td>
</tr>
<tr>
<td>Crypt dilation/distortion</td>
<td>0.5 (0 – 2)</td>
<td>1 (0 – 3)</td>
<td>1 (0 – 2)</td>
<td>0 (0 – 2)</td>
<td>1 (0 – 3)</td>
<td>1 (0 – 3)</td>
<td>1 (0 – 3)</td>
<td>1 (0 – 3)</td>
</tr>
<tr>
<td>Fibrosis/atrophy</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 1)</td>
<td>0 (NR)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 2)</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>1 (0 – 2)</td>
<td>1 (0 – 3)</td>
<td>1 (0 – 2)</td>
<td>1 (0 – 2)</td>
<td>1 (0 – 3)</td>
<td>2 (0 – 3)*</td>
<td>1 (0 – 3)</td>
<td>2 (0 – 3)*</td>
</tr>
<tr>
<td>Goblet cell number</td>
<td>0 (0 – 2)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 1)</td>
<td>0 (0 – 2)</td>
</tr>
<tr>
<td><strong>Autolysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Kittens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Additional notes for Table 4:
- The table includes lesion scores for various types of lesions, including microscopic and gross scores.
- EPEC (+) and EPEC (-) indicate positive and negative cultures for EPEC.
- The table also includes a category for mortality associated with diarrhea.
- The table concludes with a section for all kittens, showing overall lesion scores.

75
Data presented as number (No.) and percent (%) of kittens, or median (range). NR = no range. One kitten with no diarrhea cultured EPEC but eae was not amplified. Because culture results are more specific for the diagnosis of EPEC, this kitten was included in the EPEC positive group. Proportions compared between groups by Fisher Exact test. Scores compared between groups by Mann-Whitney Rank sum test
* P <0.05, ** P<0.01, *** P<0.001
Table 5. Results of parasitological examination(s) of gastrointestinal samples obtained at the time of autopsy from 51 kittens.

<table>
<thead>
<tr>
<th>Parasite identified</th>
<th>No. (%) of kittens</th>
<th>No diarrhea (n=20)</th>
<th>Diarrhea-associated mortality (n=31)</th>
<th>Gross examination</th>
<th>Dissection microscopy of GI content</th>
<th>Fecal centrifugation flotation</th>
<th>Fecal sedimentation</th>
<th>Direct microscopic examination</th>
<th>Histo-pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any parasite</td>
<td></td>
<td>15/20 (75)*</td>
<td>13/31 (42)</td>
<td>5/28 (18)</td>
<td>26/28 (93)</td>
<td>26/28 (93)</td>
<td>21/28 (75)</td>
<td>5/28 (18)</td>
<td>7/28 (25)</td>
</tr>
<tr>
<td>Toxocara cati</td>
<td></td>
<td>12/20 (60)**</td>
<td>5/31 (16)</td>
<td>3/17 (18)</td>
<td>16/18 (89)</td>
<td>11/18 (61)</td>
<td>9/18 (50)</td>
<td>1/18 (6)</td>
<td>1/18 (6)</td>
</tr>
<tr>
<td>Isospora spp.#</td>
<td></td>
<td>7/20 (35)</td>
<td>7/31 (23)</td>
<td>0/14 (0)</td>
<td>0/14 (0)</td>
<td>14/14 (100)</td>
<td>10/14 (71)</td>
<td>4/14 (29)</td>
<td>6/14 (43)</td>
</tr>
<tr>
<td>Diplydium caninum</td>
<td></td>
<td>2/20 (10)</td>
<td>2/31 (6)</td>
<td>2/4 (50)</td>
<td>4/4 (100)</td>
<td>0/4 (0)</td>
<td>0/4 (0)</td>
<td>0/4 (0)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Ancylostoma tubaeforme</td>
<td></td>
<td>3/20 (15)</td>
<td>0/31 (0)</td>
<td>0/3 (0)</td>
<td>3/3 (100)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>1/20 (5)</td>
<td>2/31 (6)</td>
<td>0/3 (0)</td>
<td>3/3 (100)</td>
<td>1/3 (33)</td>
<td>2/3 (67)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Molineus barbatus</td>
<td></td>
<td>1/20 (5)</td>
<td>1/31 (3)</td>
<td>0/2 (0)</td>
<td>2/2 (100)</td>
<td>1/2 (50)</td>
<td>2/2 (100)</td>
<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Aonchotheca putorii</td>
<td></td>
<td>0/20 (0)</td>
<td>1/31 (3)</td>
<td>0/1 (0)</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Physalopterid</td>
<td></td>
<td>0/20 (0)</td>
<td>1/31 (3)</td>
<td>0/1 (0)</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Taenia taeniaformis</td>
<td></td>
<td>0/20 (0)</td>
<td>1/31 (3)</td>
<td>0/1 (0)</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
</tr>
</tbody>
</table>

Data are shown as number (%) of kittens.

**P<0.01; *P<0.05; Χ^2^ #Isospora spp. include: I. rivolta and/or I. felis
Table 6. Comparison of medical records data from live and deceased kittens for factors associated with positive results of EPEC culture and/or qPCR for eae.

<table>
<thead>
<tr>
<th></th>
<th>Live kittens</th>
<th></th>
<th>Deceased kittens</th>
<th></th>
<th>All kittens</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EPEC negative (n=39)</td>
<td>EPEC positive (n=22)</td>
<td>EPEC negative (n=27)</td>
<td>EPEC positive (n=27)</td>
<td>EPEC negative (n=66)</td>
<td>EPEC positive (n=49)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>6.8 ± 2.1</td>
<td>6.3 ± 2.0</td>
<td>5.4 ± 1.9</td>
<td>6.5 ± 2.2</td>
<td>6.2 ± 2.1</td>
<td>6.4 ± 2.0</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>14 males</td>
<td>10 males</td>
<td>14 males</td>
<td>13 males</td>
<td>28 males</td>
<td>23 males</td>
</tr>
<tr>
<td></td>
<td>24 females</td>
<td>10 females</td>
<td>13 females</td>
<td>14 females</td>
<td>37 females</td>
<td>24 females</td>
</tr>
<tr>
<td></td>
<td>1 undocumented</td>
<td>2 undocumented</td>
<td>1 undocumented</td>
<td>2 undocumented</td>
<td>1 undocumented</td>
<td>2 undocumented</td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td>529 (280 – 966)</td>
<td>482 (227 – 1000)</td>
<td>315 (153 – 824)</td>
<td>347 (176 – 896)</td>
<td>455 (153 – 966)</td>
<td>450 (176 – 1000)</td>
</tr>
<tr>
<td><strong>Environment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days in foster care</td>
<td>1 (0 – 42)</td>
<td>5 (0 – 28)</td>
<td>6 (0 – 37)</td>
<td>6 (0 – 21)</td>
<td>5 (0 – 42)</td>
<td>6 (0 – 28)</td>
</tr>
<tr>
<td>Weaned</td>
<td>36/39 (92)</td>
<td>22/22 (100)</td>
<td>20/27 (74)</td>
<td>22/27 (81)</td>
<td>56/66 (85)</td>
<td>44/49 (90)</td>
</tr>
<tr>
<td>Obtained from source(^a)</td>
<td>30/39 (77)</td>
<td>17/22 (77)</td>
<td>17/27 (63)</td>
<td>11/27 (41)</td>
<td>47/66 (71)</td>
<td>28/49 (57)</td>
</tr>
<tr>
<td>Obtained from source(^b)</td>
<td>9/39 (23)</td>
<td>5/22 (23)</td>
<td>10/27 (37)</td>
<td>16/27 (59)</td>
<td>19/66 (29)</td>
<td>21/49 (43)</td>
</tr>
<tr>
<td><strong>Clinical signs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of diarrhea</td>
<td>15/39 (38)</td>
<td>13/22 (59)</td>
<td>16/27 (59)</td>
<td>18/27 (67)</td>
<td>31/66 (47)</td>
<td>31/49 (63)</td>
</tr>
<tr>
<td>Decreased appetite</td>
<td>0/39 (0)</td>
<td>3/22 (14)*</td>
<td>12/27 (44)</td>
<td>12/27 (44)</td>
<td>12/66 (18)</td>
<td>15/49 (31)</td>
</tr>
<tr>
<td><strong>Medications administered</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthelmintic(s)</td>
<td>28/39 (72)</td>
<td>17/22 (77)</td>
<td>13/27 (48)</td>
<td>16/27 (59)</td>
<td>41/66 (62)</td>
<td>33/49 (67)</td>
</tr>
<tr>
<td>Antiprotozoal(s)</td>
<td>21/39 (54)</td>
<td>16/22 (73)</td>
<td>12/27 (44)</td>
<td>14/27 (52)</td>
<td>33/66 (50)</td>
<td>30/49 (61)</td>
</tr>
<tr>
<td>Antibiotic(s)</td>
<td>3/39 (8)</td>
<td>2/22 (9)</td>
<td>4/27 (15)</td>
<td>8/27 (30)</td>
<td>7/66 (11)</td>
<td>10/49 (20)</td>
</tr>
<tr>
<td>Flea/tick preventative(s)</td>
<td>6/39 (15)</td>
<td>5/22 (23)</td>
<td>9/27 (33)</td>
<td>10/27 (37)</td>
<td>15/66 (23)</td>
<td>15/49 (31)</td>
</tr>
<tr>
<td>Probiotic</td>
<td>10/39 (26)</td>
<td>8/22 (36)</td>
<td>4/27 (15)</td>
<td>6/27 (22)</td>
<td>14/66 (21)</td>
<td>14/49 (29)</td>
</tr>
<tr>
<td><strong>Other treatments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous or IV fluids</td>
<td>3/39 (8)</td>
<td>7/22 (32)*</td>
<td>6/27 (22)</td>
<td>11/27 (41)</td>
<td>9/66 (14)</td>
<td>18/49 (37)**</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>0/39 (0)</td>
<td>0/22 (0)</td>
<td>2/27 (7)</td>
<td>7/27 (26)</td>
<td>2/66 (3)</td>
<td>7/49 (14)</td>
</tr>
</tbody>
</table>

Data are shown as number (%) of kittens, mean ± SD, and median (range).

\(^a\) County Animal Center

\(^b\) County SPCA
Proportions compared between groups by $X^2$ or Fisher exact test (when values < 5)

* $P < 0.05$, ** $P < 0.01$
Supplementary Table 1. Most common gross lesions observed at autopsy in 39 kittens and corresponding light microscopic lesions observed in the same region of intestine.

<table>
<thead>
<tr>
<th>Gross lesion</th>
<th>Histopathologic lesion</th>
<th>No diarrhea (n=7)</th>
<th>Diarrhea-associated mortality (n=32)</th>
<th>Total (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperemia (n=21)</td>
<td>Inflammatory infiltrate</td>
<td>2/5 (40)</td>
<td>9/16 (56)</td>
<td>11/21 (53)</td>
</tr>
<tr>
<td></td>
<td>Expanded crypts with debris</td>
<td>0/5 (0)</td>
<td>7/16 (44)</td>
<td>7/21 (33)</td>
</tr>
<tr>
<td></td>
<td>Villus blunting</td>
<td>0/5 (0)</td>
<td>6/16 (38)</td>
<td>6/21 (29)</td>
</tr>
<tr>
<td></td>
<td>Congestion</td>
<td>0/5 (0)</td>
<td>6/16 (38)</td>
<td>6/21 (29)</td>
</tr>
<tr>
<td></td>
<td>Autolysis</td>
<td>3/5 (60)</td>
<td>3/16 (19)</td>
<td>6/21 (29)</td>
</tr>
<tr>
<td></td>
<td>No specific lesion</td>
<td>1/5 (20)</td>
<td>3/16 (19)</td>
<td>4/21 (19)</td>
</tr>
<tr>
<td>Thickened wall (n=6)</td>
<td>Epithelial cell loss</td>
<td>0/1 (0)</td>
<td>3/5 (60)</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td></td>
<td>Inflammatory infiltrate</td>
<td>0/1 (0)</td>
<td>3/5 (60)</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td></td>
<td>Autolysis</td>
<td>1/1 (100)</td>
<td>1/5 (20)</td>
<td>2/6 (33)</td>
</tr>
<tr>
<td></td>
<td>No specific lesion</td>
<td>0/1 (0)</td>
<td>1/5 (20)</td>
<td>1/6 (17)</td>
</tr>
<tr>
<td>Dilation (n=7)</td>
<td>No specific lesion</td>
<td>0 (0)</td>
<td>4/7 (57)</td>
<td>4/7 (57)</td>
</tr>
<tr>
<td></td>
<td>Epithelial cell loss</td>
<td>0 (0)</td>
<td>2/7 (29)</td>
<td>2/7 (29)</td>
</tr>
<tr>
<td></td>
<td>Villus blunting</td>
<td>0 (0)</td>
<td>1/7 (14)</td>
<td>1/7 (14)</td>
</tr>
<tr>
<td></td>
<td>Inflammatory infiltrate</td>
<td>0 (0)</td>
<td>1/7 (14)</td>
<td>1/7 (14)</td>
</tr>
<tr>
<td></td>
<td>Expanded crypts with debris</td>
<td>0 (0)</td>
<td>1/7 (14)</td>
<td>1/7 (14)</td>
</tr>
<tr>
<td>Pale/pallor (n=2)</td>
<td>Inflammatory infiltrate</td>
<td>0 (0)</td>
<td>1/2 (50)</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td></td>
<td>Epithelial cell loss</td>
<td>0 (0)</td>
<td>1/2 (50)</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td></td>
<td>No specific lesion</td>
<td>0 (0)</td>
<td>1/2 (50)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Intussusception (n=1)</td>
<td>Severe coagulative necrosis of intussusceptum</td>
<td>0 (0)</td>
<td>1/1 (100)</td>
<td>1/1 (100)</td>
</tr>
</tbody>
</table>

Data represent number (No) and percent (%) of kittens. Kittens often had more than one gross and/or microscopic lesion.
Supplementary Table 2. Detailed list of medications and treatments administered to kittens in the study.

<table>
<thead>
<tr>
<th>Medication administered</th>
<th>Live kittens</th>
<th>Deceased kittens</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No Diarrhea</td>
<td>Diarrhea (n=28)</td>
</tr>
<tr>
<td></td>
<td>(n=33)</td>
<td></td>
</tr>
<tr>
<td><strong>Anthelmintic(s)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrantel</td>
<td>10 (30)</td>
<td>23 (82)</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>6 (18)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Selamectin</td>
<td>6 (18)</td>
<td>5 (18)</td>
</tr>
<tr>
<td><strong>Anti-protozoal(s)</strong></td>
<td>11 (33)</td>
<td>23 (82)</td>
</tr>
<tr>
<td>Ponazuril</td>
<td>11 (33)</td>
<td>23 (82)</td>
</tr>
<tr>
<td>Sulfadimethoxine</td>
<td>0 (0)</td>
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</tr>
<tr>
<td><strong>Antibiotic(s)</strong></td>
<td>1 (3)</td>
<td>4 (14)</td>
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<tr>
<td>Metronidazole</td>
<td>0 (0)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (3)</td>
<td>2 (7)</td>
</tr>
<tr>
<td><strong>Flea/tick preventative</strong></td>
<td>6 (18)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Selamectin</td>
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<td>5 (18)</td>
</tr>
<tr>
<td>Fipronil</td>
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</tr>
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<td>Nitenpyram</td>
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</tr>
<tr>
<td><strong>Probiotic</strong>a</td>
<td>2 (6)</td>
<td>16 (57)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous or IV fluids</td>
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<td>Mirtazapine</td>
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</tr>
<tr>
<td>Nutritional supplementsf,g</td>
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<td></td>
</tr>
</tbody>
</table>

Data shown as number and (%) of kittens

*a* Fortiflora, Nestlé Purina, Vevey, Switzerland  
*b* Hi-Vite, Vetoquinol USA Inc, Fort Worth, TX 76137  
*c* FeloVite, Vetoquinol USA Inc, Fort Worth, TX 76137  
*d* Lixotinic, Zoetis, Parsippany, New Jersey 07054  
*e* B-complex  
*f* Nutri Cal, Tomlyn/Vetoquinol USA Inc, Fort Worth, TX 76137  
*g* Glucose
Figures

**Figure 1.** Quantitative PCR (qPCR) amplification of enterocyte attaching and effacing (*eae*) gene from fecal DNA of 61 live kittens with and without diarrhea. (A) Standard curve obtained by inoculating serial dilutions of counted colony forming units (CFU) of EPEC into 100 mg aliquots of cat feces followed by DNA extraction and qPCR amplification of *eae*. Graph represents 3 independent experiments each performed in triplicate at each dilution. (B) Bar graph representation of the number of live kittens with and without diarrhea from which *eae* was amplified from fecal DNA by qPCR. (C) Individual comparative threshold (Ct) values for qPCR amplification of *eae* from fecal DNA of live kittens with and without diarrhea. The black circles represent kittens with amplification of *eae* only, whereas the white circles represent kittens that had amplification of *eae* and concurrent positive culture results for EPEC. Whisker box plots represent the median (faint line), mean (dense line), 25th percentile (bottom of the box), 75th percentile (top of the box), the 25th percentile minus 1.5x the interquartile range (bottom whisker), and the 75th percentile plus 1.5x the interquartile range (top whisker). The interquartile range (IQR) is the length of the box.
Figure 2. Quantitative PCR (qPCR) amplification of enterocyte attaching and effacing (*eae*) gene from fecal DNA of apparently healthy kittens euthanized by animal control and kittens that died or were euthanized because of severe diarrhea while in foster care. (A) Bar graph representation of the number of deceased kittens with and without diarrhea from which *eae* was amplified from fecal DNA by qPCR. (B) Individual comparative threshold (Ct) values for qPCR amplification of *eae* from fecal DNA of deceased kittens with and without diarrhea. The mean *eae* Ct value from kittens that died or were euthanized due to diarrhea was significantly lower than the mean from apparently healthy kittens indicating a greater quantity of *eae* in kittens that died or were euthanized due to diarrhea. *P*=0.02, Students t-test. (See Figure 1 legend for explanation of data points and whisker box plots).
**Figure 3.** Small intestinal and colonic histopathology lesion scores based on results of fecal culture and qPCR for *eae* in apparently healthy kittens and those dying or euthanized due to severe diarrhea. Kittens with EPEC that died with diarrhea had significantly higher small intestinal (A) and colonic (B) histopathology lesion scores compared to kittens with other causes of diarrhea and apparently healthy kittens with and without EPEC. **P<0.01, Mann-Whitney Rank sum test.**
Figure 4. Representative photomicrographs of lesions significantly associated with presence of EPEC (culture and/or eae qPCR) in kitten. Panel A: Photomicrograph of small intestine from a kitten with EPEC demonstrating: 1) epithelial cell loss and 2) inflammatory infiltrate composed of lymphocytes and plasma cells in the lamina propria. Panel B represents an increased lesion severity. Also noted in this section are crypt dilation and villus blunting in the small intestine. Panel C: Inflammatory infiltrate in the lamina propria of the colon along with crypt dilation in a kitten with EPEC. Panel D demonstrates severe inflammation composed predominantly of neutrophils obscuring crypts and architecture of the colon. Hematoxylin and eosin stain. Bar = 500 µm (Panels A-C) and 200 µm (Panel D)
Figure 5. Representative fluorescence microscopy images of focally adherent *E. coli* in the small intestine of one kitten (A – D) and the colon of another kitten (E – H) that were euthanized due to severe diarrhea. Panels A and E demonstrate the appearance of enteroadherent bacteria with Giemsa stain. Fluorescence *in situ* hybridization was performed using oligonucleotide probes specific for eubacterial 16S (Eub338; 3’ 6-FAM green) (B and F) and specific for *E. coli/Shigella* (5’ Cy3 red) (C and G). Panels D and H show overlay of fluorescence signals. Specimens were nuclear counterstained with DAPI (blue).
Supplementary Figure 1. Representative photographs of common gross lesions identified in kittens that died or were euthanized due to diarrhea. Panel A is a photograph of jejunum demonstrating the gross lesion of hyperemia. Panel B demonstrates a dilated, hyperemic jejunum and Panel C of the same section when opened demonstrates clear fluidic content and fibrin covered ulcers. Panel D demonstrates a small intestine with thickened wall. Panel E demonstrates a colon filled with melena.
Supplementary Figure 2. Representative photograph and photomicrographs of commonly identified parasites. Panel A demonstrates the gross appearance of ascarids (*Toxocara cati*) within the small intestine. Panel B demonstrates the anterior of the ascarid *Toxocara cati*, with its characteristic fleshy lips and cervical alae (Bar = 200 μm). Panel C demonstrates the anterior of a hookworm (*Ancylostoma tubaeforme*) with its characteristic dorsal mouth and buccal capsule (Bar = 200 μm). Panel D demonstrates the appearance of *Isospora felis* oocysts using flotation method (Bar = 200 μm). Panel E demonstrates the appearance of unsporulated oocysts and schizonts of *Isospora sp.* within the villi of a kitten (Bar = 100 μm).
CHAPTER III

Genomic similarity of atypical EPEC from kittens and children and bacterial factors associated with outcome of infection

Victoria E. Watson¹, Megan E. Jacob², Johanna R. Elfenbein¹, Tracy H. Hazen³, David A. Rasko³, Jody L. Gookin¹#

¹Department of Clinical Sciences and ²Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA; ³University of Maryland School of Medicine, Baltimore, Maryland, USA
Abstract

Infectious diarrhea is a leading cause of death in children and kittens. In children, enteropathogenic *Escherichia coli* (EPEC) is a common cause of diarrhea and is associated with increased risk of mortality. We demonstrated that kittens dying due to severe diarrhea have colonization of the intestinal epithelium by EPEC. The burden of EPEC was significantly associated with diarrhea-related mortality in kittens. We hypothesized that kittens carry EPEC comparable to EPEC isolated from children and phenotypic or genotypic characteristics could be used to differentiate diarrheagenic from non-diarrheagenic EPEC. In this study, EPEC were cultured from shelter kittens and sequenced for genomic analysis. Kitten EPEC were phylogenetically compared to EPEC isolated from children from the Global Enteric Multicenter Study and reference strains of *E. coli* and commensal *E. coli*. EPEC from kittens were further genotypically and phenotypically characterized. Kitten EPEC were genomically indistinguishable from EPEC from children. Methods to identify genotypic or phenotypic differences were unable to distinguish diarrheagenic from non-diarrheagenic EPEC. As has been similarly demonstrated in EPEC from children, isolates of EPEC from kittens are genotypically and phenotypically diverse. Kittens represent a reservoir species of EPEC and may augment zoonotic transmission of EPEC. Furthermore, naturally occurring EPEC infection in kittens may represent a novel animal model for study of EPEC infection in children.
Introduction

Diarrhea is currently the second leading cause of death in children under 5 years of age worldwide. Recent studies focused on infectious causes of diarrhea have identified enteropathogenic *Escherichia coli* as a leading cause of diarrhea and associated mortality in children (Kotloff et al., 2013; Pires et al., 2015). Diarrhea caused by EPEC infection afflicts approximately 17 million children each year (Kirk et al., 2015), resulting in over 120,000 fatalities (Kirk et al., 2015; Pires et al., 2015). Compared to other diarrheal pathogens, EPEC is associated with higher incidences of fatality in children with diarrhea. In one study in which 133 developing countries were assessed for the prevalence of nine diarrheal pathogens, EPEC was associated with one of the highest mortality rates (Pires et al., 2015) and in infants under 11 months of age with diarrhea, EPEC infection was associated with a 2.6 fold higher hazard of death (Kotloff et al., 2013).

Enteropathogenic *E. coli* is an attaching and effacing pathogen that adheres to intestinal epithelial cells and alters their function. Adherence of EPEC is mediated by the adhesin, intimin, which is encoded by the *eae* gene on the locus of enterocyte effacement (Jerse et al., 1990). Once intimately adhered to the intestinal epithelial cell, EPEC injects virulence genes into the host cell that increase epithelial permeability, block electrolyte transport, and lead to malabsorption (Dean and Kenny, 2004; Dean et al., 2006; Gill et al., 2007; Hecht et al., 2004; McNamara et al., 2001; Shaw et al., 2005; Shifflett et al., 2005; Simonovic et al., 2000; Thanabalasuriar et al., 2010; Tomson et al., 2005). While the cellular mechanisms of EPEC pathogenicity *in vitro* are well-characterized, children appear to have differences in susceptibility to EPEC-induced diarrhea for reasons that remain poorly
understood (Alikhani et al., 2006; Barletta et al., 2011; de Moura et al., 2012; Donnenberg et al., 2015; Hazen et al., 2016). Recent phylogenetic studies have identified that EPEC from children are highly diverse and multiple genes and gene clusters are associated with increased severity of disease (Donnenberg et al., 2015; Hazen et al., 2016). For example, a recent investigation of EPEC isolates from children with differing severities of diarrhea identified that genes for the plasmid of typical EPEC, genes of flagellin, and an allele of NleG were more prevalent in EPEC from children with fatal disease (Hazen et al., 2016). Likewise, other studies have identified an association between diarrhea severity and the presence of an EPEC pathogenicity island (O-122) containing multiple virulence factors (Afset et al., 2006; Mercado et al., 2016; Vieira et al., 2010a). In addition to differentiating pathogenic versus non-pathogenic EPEC, genotypic comparisons of EPEC isolates allow for improved methods to characterize environmental isolates and to identify carrier species capable of EPEC transmission.

Although infrequently isolated from food and companion animals, EPEC is often considered non-pathogenic in animals (Kolenda et al., 2015; Krause et al., 2005; Martins et al., 2016; Moxley and Francis, 1986). However, we discovered an association between colonization of the intestinal epithelium by enteroadherent *E. coli* and death or euthanasia due to severe illness in kittens (Ghosh et al., 2013). We recently reported a high prevalence of naturally-occurring EPEC infection in kittens in the United States. In kittens, as in children, there was a significant association between the burden of EPEC infection and diarrhea-related mortality. Kittens with EPEC demonstrated significantly greater inflammatory lesions in the intestinal tract. Although cats have been postulated to be a
reservoir species in previous studies (Goffaux et al., 2000; Krause et al., 2005; Morato et al., 2009; Pospischil et al., 1987), we have shown, in kittens, the presence of EPEC is associated with diarrhea-related mortality and intestinal pathology. Similarly to children, EPEC can be identified in clinically normal kittens and kittens with diarrhea.

The purposes of this study were to determine any genomic similarity between kitten EPEC and EPEC isolates from childhood cases of EPEC infection, and determine if naturally occurring isolates of EPEC from kittens with asymptomatic infection could be differentiated genotypically or phenotypically from EPEC isolated from kittens with lethal infection. Our hypothesis was that EPEC from kittens are genotypically similar to EPEC from children, and therefore any identified specific characteristics in EPEC from kittens with diarrhea may similarly be present in EPEC from children with diarrhea. Identification of genes, patterns of genes, or phenotypic characteristics specific to EPEC isolated from kittens with diarrhea may elucidate novel mechanisms of disease and determine new methods to identify truly pathogenic EPEC from kittens and children.
Materials and Methods

**Bacterial isolates.** Isolates of atypical EPEC were cultivated from feces collected post-mortem from apparently healthy kittens after euthanasia by animal control or from kittens that died or were euthanized due to severe diarrhea. Colonic contents were aseptically swabbed and streaked onto MacConkey agar and cultured at 37°C for isolation of Gram-negative bacteria. Bacteria were identified as *E. coli* based on the presence of indole reactivity and lack of pyrrolidonyl arylamidase and oxidase reactions using commercially available assays (Beckton, Dickinson and Company, Franklin Lakes). Isolates of *E. coli* were frozen in Lysogeny/Luria-Bertani broth (LB)-glycerol at -80°C. Individual isolates of *E. coli* were tested by means of multiplex PCR (Pennsylvania State University *E. coli* Reference Center, University Park, PA) for the presence of virulence genes encoding intimin (*eae*), Shiga toxins 1 and 2 (*stx1, stx2*), heat-stable and heat-labile toxins (*STA, STB, LT*), invasion plasmid antigen H (*ipaH*), enteroaggregative gene (*Eagg*), and cytotoxic necrotizing factors 1 and 2 (*cnf1, cnf2*) using published protocols (DebRoy and Maddox, 2001). Isolates of *E. coli* that were positive for *eae* underwent conventional PCR for identification of bundle forming pilus (*bfp*) as previously described (Gunzburg et al., 1995). Confirmation that *eae*-positive isolates were *E. coli* was performed using MALDI-TOF (Vitek® MS, Biomérieux, Marcy L’Etoile, France).

**Serotyping.** Serotyping of the O antigen was performed using antisera directed to all identified O antigens (O1-O187) (Orskov et al., 1977). H-typing was performed by PCR amplification of the *fliC* (flagella) gene followed by analysis of *HhaI* restriction fragment
length polymorphism (Pennsylvania State University *E. coli* Reference Center, University Park, PA) as previously described (Machado et al., 2000).

**Pulsed-field gel electrophoresis.** Pulsed-field gel electrophoresis was performed as described for pulsenet analysis of *E. coli*, *Shigella*, and *Salmonella* (Ribot et al., 2006). Overnight cultures of EPEC were equilibrated in cell suspension buffer solution to an absorbency of 1.08 - 1.1 at 610 nm wavelength and mixed with 1% Seakem gold agarose (Thermo Fisher Scientific, Waltham, MA) for plug formation. The plugs were sectioned to an equal size with a 2-mm width and then individually digested with restriction enzyme *XbaI* (New England Biolabs, Ipswich, MA). Plugs were embedded into an agarose gel and restriction fragments were separated by electrophoresis at 6 volts/cm for 19 hours. Standardization across gels was confirmed by including *Salmonella enterica* serotype Braenderup H9812 digested with *XbaI* in each gel. A non-pathogenic *E. coli* (ATTC 25922) (American Type Culture Collection, Manassas, VA) and EPEC strain E2348/69 (from the laboratory of JRE) were also included for PFGE pattern comparison. Gels were stained with a commercially available DNA stain (GelRed, Biotium, Fremont, CA) following the manufacturer’s protocol and imaged with a UV imager (Bio-Rad, Hercules, CA). Band patterns produced by PFGE were evaluated with BioNumerics software (Applied Maths Inc, Austin, TX).

**Detection of plasmids.** Plasmids were extracted from overnight cultures of EPEC following the alkaline lysis protocol (Ryan et al., 2000). Bacteria in LB were pelleted by centrifugation at 10,000 × g. Buffer solutions for resuspension, lysis, and neutralization were applied according to manufacturer instructions (Qiagen, Germantown, MD). The final pellet
containing plasmid DNA was respuspended in Tris-EDTA buffer and separated by gel electrophoresis in a 0.8% gel run at 110 V for 2 hours. Plasmid DNA was visualized using GelRed (Biotium, Fremont, CA) and imaged with a UV imager (UVP LLC, Upland, CA).

**Phylogenomic analyses.** Six EPEC from kittens without diarrhea and 6 EPEC from kittens with diarrhea were selected for phylogenomic analysis. Genomic DNA was isolated from each strain by growing a single colony overnight in LB medium at 37° C with shaking. The genomic DNA was isolated using the GenElute Genomic kit (Sigma-Aldrich, Corp., St. Louis, MO). Sequence and assembly-alignment were performed, using Illumina sequencing as previously described (Hazen et al., 2016; Sahl et al., 2011). The 12 kitten EPEC genomes in this study were compared with previously sequenced childhood EPEC, reference strains of typical and atypical EPEC, and selected non-EPEC *E. coli* by whole-genome phylogenomic analysis as previously described (Hazen et al., 2016).

**Gene alignments and phylogenetic analyses.** Nucleotide sequences were aligned in MEGA5 using the ClustalW algorithm as previously described (Tamura et al., 2011; Thompson et al., 1994). A maximum-likelihood phylogeny was constructed using the Kimura two-parameter model of distance estimation with 1,000 bootstrap replications (Kimura, 1980).

**BLAST score ratio analysis.** The presence or absence of known virulence-associated genes in the genome sequences generated in this study was determined using BLAST score ratio (BSR) analysis as previously described (Hazen et al., 2016; Hazen et al., 2013; Rasko et al., 2005; Sahl et al., 2014). Protein-encoding genes that were considered present and with significant similarity had BSR values above 0.8 while those with BSR values < 0.8 but ≥ 0.4
were considered to be present but divergent. The level of similarity of protein-encoding genes was compared across genomes using a large-scale BLAST score ratio (LS-BSR) analysis as previously described (Hazen et al., 2016; Sahl et al., 2014). Multiple coding sequences were analyzed across 12 kitten EPEC, 23 human/childhood EPEC, and 7 reference strains. This method utilized the same approach as for BSR and resulted in a matrix containing the sequenced gene(s) or gene cluster and the LS-BSR value. A value above 0.8 indicated conserved genome content.

**Virulence gene PCR.** An isolated colony of EPEC was mixed in 100 µl nuclease-free water and heated at 100°C for 30 minutes to liberate DNA. PCR amplification of virulence associated genes spanning the locus of enterocyte effacement (LEE) were selected, including an ATPase of the type III secretion system (T3SS) (*EscN*), a secreted protein (esp) associated with extension of the T3SS (*EspA*), and the LEE regulator (*Ler*). Also selected for PCR was the non-LEE gene lymphocyte inhibitory factor (*LifA*) (otherwise known as enterohemorrhagic *E. coli* (EHEC) factor for adherence (*efa1*)) (Afset et al., 2006; Badea et al., 2003; Deacon et al., 2010; Klapproth et al., 2005; Kobayashi et al., 2001). All conventional PCR assays were performed using previously published primer sequences and reaction conditions (Kobayashi et al., 2001; Kyaw et al., 2003; Narimatsu et al., 2010; Sharma and Zuerner, 2004). PCR products were isolated by electrophoresis on 1.5% agarose gels stained with either ethidium bromide or commercially available DNA stain (GelRed, Biotium, Fremont, CA) and visualized using a UV imager (Bio-Rad, Hercules, CA or UVP LLC, Upland, CA).
**Biofilm formation.** An *in vitro* biofilm formation assay was performed with a biofilm-forming EPEC strain incubated in 3 different growth media including Lysogeny Broth (LB) without salt, low salt (5 g/L NaCl) LB, regular (10 g/L NaCl) LB, and Todd Hewitt Broth (THB) media. Biofilm assays were performed on 96-well polystyrene suspension culture plates (Olympus plastics, Genesee Scientific, San Diego, CA) as previously described (O'Toole, 2011). Overnight cultures of EPEC were inoculated into wells containing media at a 1:100 dilution and incubated statically at room temperature (~21°C) for 72 hours. Media and planktonic bacteria were removed by rinsing 4 times and plates were dried prior to adding 0.1% crystal violet for 10 minute incubation. Plates were rinsed 5 times to remove the crystal violet solution and dried overnight. Crystal violet retained within the biofilm was solubilized in 30% acetic acid for 5 minutes and quantified by absorption at 550 nm in a BioTek Synergy 2 microplate reader (Biotek US, Winooski, VT). Each experimental run included media only wells, typical EPEC (strain E2348/69), non-pathogenic *E. coli* (ATTC 25922), and selected strains of kitten EPEC. Each isolate was assayed in quadruplicate for each experiment and each biofilm assay was repeated three times.

**Motility assay.** Motility assays were performed as previously described (Maes et al., 2013; Young et al., 1999). Each EPEC isolate was cultured overnight, then subcultured at a dilution of 1:100, and normalized to an OD600 using sterile PBS. Five µl of each normalized EPEC solution was inoculated onto 0.3% agar plates and incubated at 37°C for 5 hours. At 5 hours, the diameter of bacterial spread extending from the site of EPEC inoculation was measured. Each isolate was assayed on multiple plates per experiment and experiments were repeated 3 times. Isolates were considered non-motile when the diameter of spread was < 6 mm, poorly
motile when 6 – 9 mm, moderately motile when 9 – 11 mm, and highly motile when > 11 mm.

**Fluorescence actin-staining test.** *In vitro* adherence of kitten EPEC to HEp-2 cells (ATCC ® CCL23™) was performed as previously described (Cravioto et al., 1979; Knutton et al., 1989; Nakazato et al., 2004). HEp-2 cells were cultured in modified Eagle’s media with 10% fetal bovine serum and no antibiotics. HEp-2 cells were seeded into 4-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Fisher Scientific, Waltham, MA) and cultured for 48 hours prior to inoculation with EPEC at an MOI of 10:1. Each experiment included a chamber inoculated with a positive control typical EPEC (E2348/69), a chamber inoculated with a negative control *E. coli* (ATCC 25922), and a chamber containing only media. FAS was performed in triplicate for each isolate using the remaining 3 chambers of each slide. Chamber slides were incubated at 37°C for 4 hours, media and unattached bacteria were removed, and chambers were washed with sterile PBS. Cells were fixed in 4% formalin for 15 minutes and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO). Fluorescein isothiocyanate (FITC) bound to phalloidin (Vector Laboratories, Inc., Burlingame, CA) was added to each chamber at a concentration of 7 µg/ml and chambers were incubated for 30 minutes at 21°C in the dark. Slides were washed with PBS, dried, and nuclear counter-stained with fluorescent 4’,6-Diamidine-2’-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich Corp., St. Louis, MO). Slides were examined for adherence using fluorescence microscopy (Leica Microsystems Inc., Buffalo Grove, IL).
**Statistical analysis.** Data were tested for significant differences in distributions of observations between EPEC isolates using Fisher exact test and odds ratio. Differences in the mean or median values of continuous data were analyzed using student’s T test or Mann-Whitney rank sum test, respectively. Analyses were performed using commercial software (Sigmaplot 12; Systat Software, Inc., San Jose, CA) and data were considered significant when $P < 0.05$. 
Results

Genotypic characterization of Kitten EPEC isolates.

Eight EPEC isolates from 5 apparently healthy kittens (asymptomatic infection, AI) and 17 EPEC from kittens that died or were euthanized due to severe diarrhea (lethal infection, LI) were studied. All isolates of kitten EPEC were identified as atypical EPEC based on the presence of eae and absence of the genes encoding Shiga toxins 1 and 2 and bundle forming pili (Table 1).

Kitten EPEC were represented by diverse serotypes. Eight different combinations of O serotypes and H types were observed among the EPEC isolates (Table 2). More than one serotype of EPEC was isolated from 1/5 AI kittens and 2/6 LI kittens. Isolates with H21 were significantly associated with a lethal outcome of infection (Fisher exact test; P=0.022). A single isolate of each identified serotype from each kitten was selected for further examination of their genotypic diversity using PFGE.

Kitten EPEC are genotypically diverse. The isolates were observed to be genetically diverse (Figure 1) and no significant clustering was observed between AI and LI pulsotypes. All isolates of kitten EPEC were determined to carry multiple plasmids ranging in size from approximately 4,000 base pairs to > 10,000 base pairs (Figure 2). There was no distinct pattern of plasmid profiles of among AI isolates compared to LI isolates.

Phylogenomic analysis of kitten EPEC with comparison to GEMS human EPEC isolates.

To compare the genomic similarity of kitten EPEC to previously characterized isolates of EPEC, we sequenced the genomes of 12 kitten EPEC isolates. Using the phylogenetic
information included in the core genome alignment of the 12 kitten EPEC genomes and genomes from previously defined *E. coli* phylogenetic groups, the majority of kitten EPEC clustered in lineages previously designated as EPEC2 and EPEC7 (Figure 3). Based on core genome content, no differences were demonstrated between kitten EPEC and human EPEC. Using a large scale BLAST score ratio to analyze the total genome content of selected human EPEC isolates and all kitten EPEC isolates, no genome clusters were identified as separating kitten EPEC from human EPEC (Table 3). Likewise, no genome clusters were identified that differentiated AI versus LI kitten EPEC isolates.

**Virulence genes associated of kitten EPEC with different clinical outcomes.**

To determine the presence or absence of known virulence genes in the 12 kitten EPEC genomes sequenced in this study, we performed a BLAST score ratio analysis. The percents of kitten EPEC genomes with highly conserved virulence gene sequence identities (≥ 0.8 BSR) are shown in Figure 4. The presence of additional virulence genes not included in the BSR analysis and either spanning the composition of the locus of enterocyte effacement (LEE) or previously associated with EPEC disease outcome (LifA/efa1) were investigated by direct PCR (Table 4). BLAST score ratio analysis and direct PCR did not demonstrate significant differences in virulence gene content when comparing AI versus LI isolates. However, all LI EPEC isolates contained conserved genes within the general secretory pathway versus only 3/6 AI EPEC isolates contained these genes.

**Phenotypic characterization of kitten EPEC with different clinical outcomes.**

Virulence of EPEC could be ostensibly influenced by an ability to form transmissible biofilm, gain access to the intestinal epithelium, and establish adhesion to enterocytes.
Accordingly, a single representative isolate of each identified serotype for each kitten was assayed for biofilm formation, motility, and adherence to HEp-2 cells *in vitro*. Based on assay in LB with no salt, the majority of kitten EPEC isolates were identified as moderate biofilm formers (Figure 5). The average absorbency for EPEC isolated from apparently healthy kittens was 1.3 ± 0.2 in no salt LB and 0.5 ± 0.5 in low salt LB. The average absorbency for EPEC isolated from kittens with diarrhea was 1.4 ± 0.5 in no salt LB and 0.5 ± 0.5 in low salt LB. No significant differences were observed in magnitude of biofilm formation by AI versus LI isolates of kitten EPEC. All isolates of EPEC were determined to be motile (Figure 6). The EPEC AI isolates had an average (± SD) motility of 8.7 ± 2.0 mm, while the EPEC LI isolates had an average motility of 12.5 ± 1.5 mm. There was no significant difference in the motility of AI compared to LI isolates of kitten EPEC. Using the fluorescence actin staining (FAS) assay, we determined that none of the 4 AI isolated of kitten EPEC were capable of adherent to HEp-2 cells *in vitro*. In contrast, 4/8 (50%) of LI isolates were adherent; one EPEC displayed a localized adherence-like pattern and 3 showed a nonspecific pattern of adherence (Figure 7).
Discussion

The diversity of atypical EPEC cultured from kittens closely parallels the recently documented genomic diversity of EPEC cultured from children (Hazen et al., 2016). Genomic analysis of EPEC has been recently utilized to demonstrate genetic diversity of atypical and typical EPEC from asymptomatic children and children with diarrhea (Donnenberg et al., 2015; Hazen et al., 2016; Tennant et al., 2009a). In the genomic study by Hazen et al, 42% (10/24) of EPEC within phylogroup B1 were cultured from children that died due to diarrhea. Remarkably, six kitten EPEC clustered within EPEC phylogroup B1, 3 of which clustered with the recently characterized phylogenomic lineage EPEC 7 (Hazen et al., 2016). Therefore, kittens can shed EPEC genomically similar to EPEC associated with diarrhea and diarrhea-related mortality in children. The genomic data acquired in this study augments past studies and hypotheses regarding a feline reservoir and source of EPEC infection (Krause et al., 2005; Morato et al., 2009). The EPEC cultured from kittens in a developed country, where EPEC infection is infrequently diagnosed, are genomically similar to EPEC from sub-Saharan Africa obtained during the Global Enteric Multicenter Study. Not only does this study imply that there may be a feline reservoir, but it also indicates that EPEC, capable of causing diarrhea and death in children in developing countries, is present in the United States. Furthermore, it is highly possible that companion animals in countries where EPEC is more prevalent and disease-associated are more likely to shed EPEC and shed higher amounts of EPEC.

Most of the serotypes we identified in kittens have been previously identified as EPEC. Of the serotypes we identified in kittens with diarrhea, serotype O111 has been
identified as an EPEC, an enteroaggregative *E. coli*, and one of the non-O157 enterohemorrhagic *E. coli* (Botelho et al., 2003; Brooks et al., 2005; Chen et al., 2016; Scotland et al., 1989; Wang et al., 2016). The other serotypes designated as members of EPEC by the World Health Organization include: O26, O55, O86, O114, O119, O125, O126, O127, O128, O142, and O158 (Baliere et al., 2016). Interestingly, serotype O128, although found only in kittens without diarrhea, is an atypical EPEC or EHEC previously associated with human diarrheal disease (Tennant et al., 2009a; Whittam et al., 1993). There is little information available for the most commonly identified serotype (O153) in kittens in this study; however, O153 serotypes of EPEC have been documented (Baliere et al., 2016; Tennant et al., 2009a; Tilak and Mudaliar, 2012). In one study performed in India, serotype O153 was identified as the most prevalent serotype of enteroaggregative *E. coli* in fecal samples from children with and without diarrhea (Tilak and Mudaliar, 2012). One study that investigated the prevalence of EPEC and EHEC in French coastal areas found that serotype O153:H2 was among the serotypes of EPEC with the highest amount of virulence genes (34 out of the 75 virulence genes tested) (Baliere et al., 2016). Although kittens in this study did not shed the most common serotypes historically associated with EPEC, the diversity of serotypes identified in kittens corresponds to the diversity of serotypes of EPEC in humans and the environment.

The identification of a common non-O157 EHEC serotype, O111, as one of the EPEC found in kittens with diarrhea is of interest due to findings of conversion between EPEC and EHEC (Hazen et al., 2013; Wick et al., 2005; Zhou et al., 2010). The conversion of EHEC to atypical EPEC, by the loss of stx genes, has been documented in human patients with
gastrointestinal infections (Bielaszewska et al., 2007). This study also demonstrated that stx-encoding phages from the EHEC serotype O26 could infect atypical EPEC strains in vitro (Bielaszewska et al., 2007). Further implicating the close association between EPEC O111 and EHEC O111 is demonstrated by the close phylogenetic clustering of the O111 (Kitten/isolate 33/2) to GEMS members of the non-O157 EHEC lineage 2 on the phylogenetic tree (Figure 2). It is highly possible that kittens may not only act as a reservoir for EPEC but may also either act as a reservoir for EHEC, or at the very least carry atypical EPEC susceptible to stx gene transduction via bacteriophages.

This study demonstrated EPEC cultured from kittens are genomically diverse. Sequencing of the bacterial genomes followed by BSR and LS-BSR analyses to determine conserved genes between kitten EPEC and human EPEC demonstrated genomic diversity in kitten EPEC. Likewise, the method of PFGE showed genomic diversity and was unable to detect a PFGE pattern differentiating EPEC cultured from kittens with diarrhea from EPEC cultured from apparently healthy kittens. Furthermore, PFGE demonstrated that kittens are not being infected by the same or similar pulsotype; there is no evidence of an outbreak. Although kitten EPEC contain multiple plasmids, there was no specific plasmid pattern on gel electrophoresis associated with EPEC from kittens with diarrhea.

In the study by Hazen et al, they proposed that certain genomic features are needed for greater pathogenicity of EPEC (Hazen et al., 2016). Genes associated with higher disease severity outcome included those encoding flagellin, the EAF plasmid, bacteriophage-related proteins, a protein within the LPS synthesis pathway, capsule transporter protein, and hypothetical proteins (Donnenberg et al., 2015; Hazen et al., 2016). Additionally, an allele of
the effector NleG was identified in a greater number of EPEC from cases of mortality (Donnenberg et al., 2015; Hazen et al., 2016). Interestingly, one of the nleG sequences from the EPEC strain B171 was conserved in 67% (8/12) of the EPEC isolated from kittens. However, the sequence (cluster 6826) of nleG associated with symptomatic to lethal disease due to typical EPEC was not present in kitten EPEC (Donnenberg et al., 2015). Comparing 6 EPEC from kittens without diarrhea to 6 EPEC from kittens with diarrhea did not demonstrate genes associated with disease outcome. The small number of kitten isolates in this study represents a limitation of the study; however, the data demonstrate a novel parallel between kitten EPEC and EPEC from children even with the small sample size. In addition to sample size, a possible reason for not finding specific gene(s) associated with disease outcome could be the higher genomic diversity of atypical EPEC, compared to typical EPEC (Tennant et al., 2009a).

Similarly to BSR analyses and presence of virulence genes, PCR amplification of particular virulence genes occurred unpredictably in EPEC from kittens. Surprisingly, our data demonstrated espA was infrequently amplified from EPEC cultured from kittens with diarrhea. The protein, EspA, allows for transportation of virulence effectors from the T3SS into the cell and is also associated with adhesion and host immune response (Cleary et al., 2004; Durand et al., 2013; Loureiro et al., 1998; Sekiya et al., 2001). The inability to amplify espA from strains cultured from kittens with diarrhea was likely due to differences in sequences of espA among EPEC strains, as previously demonstrated in EPEC from human infections (Neves et al., 1998). The lack of amplification of espA was, therefore, unlikely to be due to an actual lack of espA, but rather non-specificity of the primers to all espA
sequences. The LifA/efa1 gene was PCR-amplified from more, although not significant, kittens with diarrhea than without. The LifA/efa1 gene has been demonstrated to be important in colonization of EHEC in calves and Citrobacter rodentium in mice and is associated with EPEC in children and adults with diarrhea but not EPEC from individuals without diarrhea (Afset et al., 2006; Deacon et al., 2010; Klapproth et al., 2005; Mercado et al., 2016; Narimatsu et al., 2010; Vieira et al., 2010a). Overall, the PCR amplification data did not determine a specific virulence gene associated with diarrhea in kittens; however, similarly to other studies, LifA/efa1 may play a role in pathogenicity of EPEC in kittens.

This study identified a wide range of variability in the phenotypic characteristics of EPEC from kittens. This study focused on phenotypic characteristics likely associated with exposure to EPEC (biofilm) and capability of EPEC to colonize intestinal epithelial cells (motility and adherence). We found no association between biofilm formation and presence of diarrhea. Due to the high number of EPEC able to form biofilm, exposure to EPEC is likely high. EPEC from kittens with diarrhea were non-significantly more motile as determined by diameter of the halo surrounding the site of EPEC inoculation on low density agar. Due to the small number of EPEC analyzed, the lack of significance (P=0.07) in the difference in motility cannot be interpreted accurately. Motility of E. coli is highly dependent on flagella and flagellin genes are associated with lethal cases of EPEC-induced diarrhea in children (Hazen et al., 2016). It is possible that a specific flagellar gene or genes produce EPEC with improved motility. This could then lead to increased pathogenicity by increasing passage from the lumen, through the mucus barrier, and access of EPEC to the intestinal epithelial cells. In addition to our data on motility of EPEC from kittens, we also showed that
EPEC from kittens without diarrhea were non-adherent as determined by FAS assay, whereas half of the EPEC from kittens with diarrhea adhered to HEp-2 cells. This study provides some evidence that EPEC leading to disease with higher severity may be more motile and more adherent.

We aimed to determine the genotypic and phenotypic diversity of EPEC from kittens with an interest on whether a specific characteristic was present in EPEC from kittens with diarrhea but not in EPEC from kittens without diarrhea. The results of this study showed that EPEC from kittens are highly diverse, paralleling data from similar studies on EPEC in children and dogs (Goffaux et al., 2000; Hazen et al., 2016). The phenotypic diversity, particularly the differences in motility, may indicate a specific phenotype of EPEC with an increased ability to cause disease. Comparably to kittens, EPEC can be cultured from both healthy children and children with diarrhea, indicating that certain host characteristics, EPEC characteristics, and/or microbiome communities are important for disease due to EPEC. Our studies on the role of EPEC in diarrhea and mortality in kittens have investigated the host characteristics and the differences in EPEC from kittens with and without diarrhea. Current and future studies will focus on the gut microbiome and investigate the interactions between members of the microbiota and EPEC that may influence disease in both kittens and children.
## Tables

Table 1. Characterizations of kitten EPEC.

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>Kitten ID</th>
<th>Isolate ID</th>
<th>Serotype</th>
<th>eae PCR</th>
<th>bfp PCR</th>
<th>PFGE</th>
<th>Plasmid(s)</th>
<th>Phylogenomic lineage</th>
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</thead>
<tbody>
<tr>
<td>AI</td>
<td>12</td>
<td>12-1</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>EPEC7</td>
</tr>
<tr>
<td>AI</td>
<td>12</td>
<td>12-3</td>
<td>O128:H2</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
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<td>25</td>
<td>25-2</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>EPEC2</td>
</tr>
<tr>
<td>AI</td>
<td>25</td>
<td>25-3</td>
<td>O128:H2</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>55</td>
<td>55-3</td>
<td>O153:H-</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>Undetermined</td>
</tr>
<tr>
<td>AI</td>
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<td>LL1</td>
<td>O88:H-</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>EPEC2</td>
</tr>
<tr>
<td>AI</td>
<td>LL10</td>
<td>LL2</td>
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<td>-</td>
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<td>+</td>
<td>EPEC2</td>
</tr>
<tr>
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<td>53</td>
<td>53-1</td>
<td>O153:H21</td>
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<td>-</td>
<td>Yes</td>
<td>+</td>
<td>Undetermined</td>
</tr>
<tr>
<td>AI</td>
<td>53</td>
<td>53-2</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
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<td>LI</td>
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<td>5-1</td>
<td>O108:H21</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>Undetermined</td>
</tr>
<tr>
<td>LI</td>
<td>5</td>
<td>5-2</td>
<td>O108:H21</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>5</td>
<td>5-3</td>
<td>O108:H21</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
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<td>31</td>
<td>31-1</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>Undetermined</td>
</tr>
<tr>
<td>LI</td>
<td>31</td>
<td>31-2</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>31</td>
<td>31-4</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>33</td>
<td>33-1</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>33</td>
<td>33-2</td>
<td>O111:H8</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>Undetermined</td>
</tr>
<tr>
<td>LI</td>
<td>33</td>
<td>33-3</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>34</td>
<td>34-1</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>EPEC7</td>
</tr>
<tr>
<td>LI</td>
<td>34</td>
<td>34-2</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>34</td>
<td>34-3</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>35</td>
<td>35-2</td>
<td>O4:H+</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>35</td>
<td>35-4</td>
<td>O153:H7</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>EPEC7</td>
</tr>
<tr>
<td>LI</td>
<td>53</td>
<td>53-1</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td>+</td>
<td>Undetermined</td>
</tr>
<tr>
<td>LI</td>
<td>53</td>
<td>53-2</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LI</td>
<td>53</td>
<td>53-3</td>
<td>O153:H21</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

AI= asymptomatic infection, LI= lethal infection, ND= not done
Table 2. Distribution of serotypes among 24 EPEC isolates from different clinical outcomes examined in the study.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Total isolates (n=24)</th>
<th>AI isolates (n=7)</th>
<th>LI isolates (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O153:H7</td>
<td>8/24 (33)</td>
<td>2/7 (28)</td>
<td>6/17 (35)</td>
</tr>
<tr>
<td>O153:H21</td>
<td>6/24 (25)</td>
<td>0/7 (0)</td>
<td>6/17 (35)</td>
</tr>
<tr>
<td>O108:H21</td>
<td>3/24 (12.5)</td>
<td>0/7 (0)</td>
<td>3/17 (18)</td>
</tr>
<tr>
<td>O128:H2</td>
<td>3/24 (12.5)</td>
<td>3/7 (43)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>O4:H+</td>
<td>1/24 (4)</td>
<td>0/7 (0)</td>
<td>1/17 (6)</td>
</tr>
<tr>
<td>O111:H8</td>
<td>1/24 (4)</td>
<td>0/7 (0)</td>
<td>1/17 (6)</td>
</tr>
<tr>
<td>O153:H-</td>
<td>1/24 (4)</td>
<td>1/7 (14)</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>O88:H-</td>
<td>1/24 (4)</td>
<td>1/7 (14)</td>
<td>0/0 (0)</td>
</tr>
</tbody>
</table>

Data presented as number (No.) and percent (%) of EPEC serotype.
Table 3. Number of gene clusters identified using LS-BSR in relation to species of origin (cat versus human) and clinical outcome.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>No. of Genome(s) (Group 1)</th>
<th>No. of Genome(s) (Group 2)</th>
<th>No. of LS-BSR Gene Clusters&lt;sup&gt;a&lt;/sup&gt;</th>
<th>All Genome&lt;sup&gt;b&lt;/sup&gt;</th>
<th>≥50% of Genome&lt;sup&gt;b&lt;/sup&gt;</th>
<th>≥1 Genome&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>LI (Cat)</td>
<td>Al (Cat)</td>
<td>6</td>
<td>6</td>
<td></td>
<td>0</td>
<td>8</td>
<td>596</td>
</tr>
<tr>
<td>Al (Cat)</td>
<td>LI (Cat)</td>
<td>6</td>
<td>6</td>
<td></td>
<td>0</td>
<td>113</td>
<td>570</td>
</tr>
<tr>
<td>Cat (LI + Al)</td>
<td>Human</td>
<td>12</td>
<td>30</td>
<td></td>
<td>0</td>
<td>25</td>
<td>581</td>
</tr>
<tr>
<td>Human</td>
<td>Cat (LI + Al)</td>
<td>30</td>
<td>12</td>
<td></td>
<td>0</td>
<td>15</td>
<td>2900</td>
</tr>
</tbody>
</table>

<sup>a</sup>The total number of core gene clusters (LS-BSR value ≥0.8) in all of the genomes (n=42) analyzed was 2,014.

<sup>b</sup>The number of gene clusters that were present in all genomes, ≥50% of the genomes, or ≥1 of the genomes of Group 1 (LS-BSR ≥0.8) and absent from all of the genomes of Group 2 (LS-BSR <0.4).
Table 4. Presence of locus of enterocyte effacement (LEE) genes, on the basis of PCR amplification, in 12 kitten EPEC isolates.

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>No. (%) EPEC isolates (n=12)</th>
<th>No. (%) AI isolates (n=4)</th>
<th>No. (%) LI isolates (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EscN</td>
<td>10 (83)</td>
<td>3 (75)</td>
<td>7 (88)</td>
</tr>
<tr>
<td>Ler</td>
<td>10 (83)</td>
<td>3 (75)</td>
<td>7 (88)</td>
</tr>
<tr>
<td>EspA</td>
<td>6 (50)</td>
<td>3 (75)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>Efa1/LifA</td>
<td>5 (42)</td>
<td>1 (25)</td>
<td>4 (50)</td>
</tr>
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</table>
Figure 1. Pulsed-field gel electrophoresis (PFGE) of 16 kitten EPEC isolates obtained from cases of asymptomatic (AI) and lethal (LI) infection. A typical EPEC strain (E2348/69) and a non-pathogenic *E. coli* strain (ATCC ® 25822™) are included for comparison. The isolates were clustered using unweighted pair group method with arithmetic mean (UPGMA) and the scale on the left represents the % similarity between each pulsotype. Isolate ID and clinical outcome for each pulsotype are shown on the right.
Figure 2. Kitten EPEC isolates contain plasmids of varying sizes. No identifiable pattern is present in the 6 EPEC from kittens with asymptomatic infection (AI) or the 8 EPEC from kittens with lethal diarrhea (LI). Typical EPEC strain E2348/69 is included for comparison. MW = 10,000 base pair molecular weight ladder.
Figure 3. Phylogenomic analysis of 12 kitten EPEC isolates associated with asymptomatic or lethal infections compared with previously sequenced EPEC genomes and diverse reference genomes of *E. coli* and *Shigella* isolates. Phylogenetic tree was produced using whole genome sequences aligned with Mugsy. Phylogenetic tree was prepared using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The reference strains, Sakai (EHEC) and E2348/69 and B171 (typical EPEC) are indicated with an asterisk. AI=asymptomatic infection, LI=lethal infection
Figure 4. Multiple virulence genes associated with pathogenicity of EPEC and/or EHEC are variably present in EPEC from kittens. The presence of each virulence gene was determined using BSR values of $\geq 0.8$ indicating high similarity to the reference strains of EPEC. The percentage of kitten EPEC (n=12) with conserved virulence genes is shown on the y-axis. Multiple sequences of NleG were tested and although no kitten EPEC had nleG sequences similar (BSR value $\geq 0.8$) to nleG from E2348/69 (*), 66.7% (8/12) contained nleG sequences similar to a sequence from B171 (#).
Figure 5. Kitten EPEC isolates form biofilm. All kitten EPEC were assessed for biofilm formation in LB with no salt. Absorbency of > 0.2 (horizontal line) was considered moderate to strong biofilm formation. Isolate numbers are noted on the x-axis. AI= asymptomatic infection; LI= lethal infection.
Figure 6. Motility assay shows a wide range of motility of kitten EPEC. Diameters of motility ranged from minimal (6 mm) to high (>10 mm). Whisker box plot representation for motility measurements of EPEC isolated from AI and LI kittens. Whisker box plots represent the median (line within box), 25\textsuperscript{th} percentile (bottom of the box), 75\textsuperscript{th} percentile (top of the box), the 25\textsuperscript{th} percentile minus 1.5x the interquartile range (bottom whisker), and the 75\textsuperscript{th} percentile plus 1.5x the interquartile range (top whisker). The interquartile range (IQR) is the length of the box. Data-points represent the average motility per isolate.
Figure 7. Fluorescence actin staining assay demonstrates epithelial cell adherence of kitten EPEC isolates. Panel A: Negative control showing HEp-2 cells with no adherent bacteria. Panel B: Positive control of typical EPEC strain E2348/69 showing localized adherence characteristic of typical EPEC. Panels C and D demonstrate adherence patterns identified in EPEC from kittens with diarrhea – localized adherence-like pattern (C) and nonspecific/indeterminate pattern (D). HEp-2 cells were counterstained with DAPI.
CHAPTER IV

Analysis of enteropathogenic Escherichia coli experimental infection in kittens with antibiotic-induced microbial disruption and benefit of a selected member of the microbiome

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Abstract

Diarrhea is responsible for the death of approximately 900,000 children per year worldwide. In children, enteropathogenic *E. coli* (EPEC) is a common cause of diarrhea and is associated with higher risk of fatality. Although atypical EPEC has been identified in animals, the disease is poorly reproduced in experimental animal models. As in children, mortality in kittens is often attributed to diarrhea and we previously identified enteroadherent EPEC in intestinal tracts of deceased kittens. Although there is a clear association between EPEC and diarrhea-related mortality in kittens, it is unknown whether EPEC is actually a primary cause of diarrhea. The role of the gut microbiota is also undetermined in regards to susceptibility to EPEC induced diarrhea in kittens. Furthermore, we have observed that a particular species of naturally-occurring enterococcus bacteria (*Enterococcus hirae*) are found more commonly in the gastrointestinal tract of kittens that do not have EPEC, implying that *E. hirae* may protect kittens from EPEC infection. The purpose of this study was to determine the effects of EPEC on intestinal function in kittens and if a probiotic was able to ameliorate disease. In this study, we have shown that EPEC affects intestinal function in kittens regardless of clinical disease. Post-infection, kittens with antibiotic-induced alterations to gut microbiota had significantly increased fecal wet weight. With the limited clinical signs noted post-infection, we did not demonstrate a significant effect of the probiotic on EPEC infection in kittens. Elucidating the mechanisms of disease due to EPEC and potential therapeutics in kittens can benefit both human and veterinary species.
Introduction

Diarrhea is responsible for the death of an estimated 900,000 children per year worldwide with the majority of mortality occurring in developing countries (Black et al., 2010; Kirk et al., 2015; Kotloff et al., 2013; Liu et al., 2012). Multiple studies have determined that diarrhea in these children can be largely attributed to a mere handful of infectious agents (Kirk et al., 2015; Kotloff et al., 2013; Pires et al., 2015). In particular, diarrhea caused by typical enteropathogenic E. coli (EPEC) is associated with a 2.6 fold higher hazard of death – the largest reported in the Global Enteric Multicenter Study (GEMS) (Kotloff et al., 2013). In addition to typical EPEC’s association with mortality, both typical and atypical EPEC (tEPEC and aEPEC) remain one of the common causes of diarrhea as they are responsible for over 81 million cases of diarrhea per year of which 17 million cases are diagnosed in children (Kirk et al., 2015). Although somewhat controversial, aEPEC identification in children with diarrhea is increasing in both developing and developed countries, leading to speculation that aEPEC is an emerging pathogen in children (Hu and Torres, 2015).

As in children, diarrheal disease and subsequent dehydration and malnutrition significantly impact neonatal and young animals, including kittens. Mortality rate estimates range from 10 to 30% for < 6-month-old kittens (Bucheler, 1999; Cave et al., 2002; Murray et al., 2008; New et al., 2004; Nutter et al., 2004; Sparkes et al., 2006). Within one shelter in Wake County, NC the kitten mortality rate from 2012-2015 ranged from 59% to 24% (personal communication, SJ Strong). Although the cause of death may remain unknown, kittens often die with diarrhea, signs of enteritis, and/or presumed or diagnosed infectious
diseases (Cave et al., 2002; Ghosh et al., 2013; Nutter et al., 2004; Sparkes et al., 2006). Our laboratory has identified EPEC infection in kittens (Ghosh et al., 2013). To further determine the role of EPEC in diarrhea and diarrhea-related mortality in kittens, we performed a case-controlled prospective study. Atypical EPEC was cultured from both kittens with and without diarrhea. Using qPCR for detection of the gene (eae) encoding the adhesin, intimin, in fecal DNA, we identified an association between diarrheal death and fecal quantity of EPEC. Although we demonstrated an association between burden of aEPEC and diarrhea-related mortality in kittens, it is unknown if aEPEC is a primary cause of diarrhea.

In children, there is some controversy over the pathogenicity of aEPEC (Hu and Torres, 2015). In particular, while some studies identify EPEC as significantly associated with diarrhea (Estrada-Garcia et al., 2009; Tilak and Mudaliar, 2012), others find that EPEC can be cultured from the feces of children with and without diarrhea at relatively similar prevalence (Barletta et al., 2011; Enserink et al., 2014; Nikbin et al., 2012). Recent studies have identified genetic differences in EPEC from asymptomatic children compared to EPEC from children with diarrhea (Afset et al., 2006; Donnenberg et al., 2015; Hazen et al., 2016; Ingle et al., 2016). Another possible difference in children with different clinical outcomes associated with EPEC could be due to difference in susceptibility to EPEC. We hypothesize that the difference defining susceptibility to disease is within the structure of the microbiome and that EPEC leads to diarrhea predominantly in hosts with altered microbial structure within the gastrointestinal tract. In our original study identifying EPEC in kittens, we demonstrated a profound shift in the mucosa-associated enterococci linked to generalized illness and the presence of enteroadherent EPEC (Ghosh et al., 2013). EPEC studies utilizing
animal models of the disease have similarly shown an association between changes in the microbiome and disease outcome (Sekirov et al., 2008; Vong et al., 2015; Wlodarska et al., 2011).

In the studies reported here, we aimed to determine if aEPEC is a primary cause of diarrhea and the effects of aEPEC infection on intestinal function in kittens. We hypothesized that in kittens, aEPEC either acts as a primary cause of diarrhea or is able to alter intestinal function leading to malnutrition and/or intensified diarrhea due to other enteric or opportunistic pathogens. Additionally, we aimed to determine if alteration of the microbiome impacts susceptibility to disease due to aEPEC in kittens. Our rationale was that identification of aEPEC as a primary cause of diarrhea in kittens could provide a unique opportunity for development of diagnostic, treatment, or prevention strategies having dual benefit to both kittens and children with aEPEC infection. One such prevention strategy involves the use of probiotics. In a previous study, we determined that terminally ill kittens have a disrupted mucosa-associated enterococcus population, whereas healthy kittens contained only Enterococcus hirae (Ghosh et al., 2013). Furthermore, E. hirae was adherent to the intestinal epithelial cells and EPEC was not documented in any kitten with enteroadherent E. hirae. Therefore, we hypothesized that E. hirae may outcompete aEPEC for adherence to enterocytes and thus could limit aEPEC effect on enterocytes and ameliorate disease. By administering a probiotic composed of E. hirae to kittens infected with aEPEC in the following studies, we aimed to determine if E. hirae benefits or protects kittens.
Materials and methods

**Kittens.** Six-week-old kittens were obtained from a commercial vendor. Individual kittens were housed in separate cages and received AAFCO-approved commercially available food (Hill’s® Science Diet kitten healthy development; Topeka, KS) *ad libitum* throughout the study. Each kitten also received ¼ teaspoon of canned cat food (Hill’s® Science Diet a/d) each morning. Kittens were housed under conditions of controlled light cycle and ambient temperature and were handled in compliance with biosafety level 2 guidelines. Kittens were acclimated to this new environment for 5 days. The study was approved by the North Carolina State University Institutional Animal Care and Use Committee and no animals were euthanized for the purpose of the study.

**Influence of treatment with antibiotics.** To determine the impact of altered gut microbiota on susceptibility to aEPEC-induced intestinal dysfunction, two separate groups of kittens were studied. The first group (no antibiotics) included eight kittens that did not receive any antibiotics prior to experimental infection with aEPEC. Following completion of the study with the first group, the second group (antibiotics) included eight kittens that were administered oral antibiotics consisting of amoxicillin/clavulanate (12.5 mg/kg twice daily) (Zoetis, Parsippany, New Jersey) and pradofloxacin (7.5 mg/kg once daily) (Bayer, Shawnee Mission, Kansas) for 5 consecutive days followed by an “antibiotic washout” period of 72 hours prior to experimental infection. The experimental time line for each group of kittens is shown in Figure 1.

**Experimental EPEC infection.** Experimental infections were performed using 3 isolates of aEPEC, each having a unique serotype and pulsotype, that were cultured from the colonic
contents of 3 kittens that died with clinical signs of diarrhea. Each isolate was identified as *E. coli* by means of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Vitek® MS; Biomérieux, Marcy l’Etoile, France) (Stephan et al., 2011). Each isolate was identified as aEPEC by demonstrating the presence of *eae* and absence of genes encoding bundle-forming pilus and Shiga-toxins 1 and 2 (DebRoy and Maddox, 2001). Each isolate was stored in 50% lysogeny broth (LB)-glycerol at -80°C. Prior to use in experimental infection, each isolate was grown overnight on LB agar at 37°C. A single colony from each plate was suspended in LB broth and grown overnight. The following morning, overnight cultures were sub-cultured at 1:25 concentration in LB and incubated on a shaker platform at 37°C until an OD$_{600}$ of 0.4 was obtained (~ 10$^8$ CFU/ml). To prepare the inoculum for each kitten, a one milliliter aliquot of each isolate (3 mls total) was mixed in a conical tube and centrifuged at 2,000 × g for 15 minutes. The supernatant was removed and the pellet was reconstituted with 100 µl of sterile water. A tandem mixture of the isolates was simultaneously prepared for serial dilutions and plate counting to confirm the CFU/ml of the inoculum used for infection. Experimental infections were achieved by adding the 100 µl aliquot of aEPEC to ¼ teaspoon of canned cat food which was offered to each kitten and promptly ingested. After determining the effects of aEPEC on kittens and prior to the end of the studies, aEPEC eradication was performed by administration of pradofloxacin (7.5 mg/kg once daily) (Bayer, Shawnee Mission, Kansas) for 7 days. Eradication was confirmed by the absence of cultivable aEPEC in the feces.

**Establishing a dose for experimental aEPEC infection.** An estimate of the inoculation dose of aEPEC to use in experimental infection was made using quantitative PCR data
obtained from amplification of \textit{eae} from the fecal DNA of naturally infected kittens and a previously developed standard curve for the relationship between \textit{eae} Ct and CFU aEPEC (Chapter II). To estimate the influence of the feline gastric environment on survival of orally-inoculated aEPEC, we additionally examined growth of the aEPEC isolates under conditions designed to simulate feline gastric contents. A mixture of the 3 different isolates of aEPEC were inoculated in duplicate into a simulated stomach (50 ml conical tube) containing 5 mls of an acidic solution. To obtain the acidic pH (pH2) and simulate ion composition, two buffers (0.2 M KCl and 0.2 M HCl) were combined. To further model a simulated stomach, 0.005 mg/ml pepsin was added to the solution. The pH, ion composition, and pepsin concentration was extrapolated from published data on feline and canine gastric contents (Arndt et al., 2013; Sutton, 2004; Vertzoni et al., 2005). To replicate the vehicle intended for use in delivery of the experimental aEPEC inoculum, \( \frac{1}{4} \) teaspoon of canned cat food (Hill’s\textsuperscript{\textregistered} Science Diet a/d) was included in each tube. The solution was incubated in a shaking water bath (30 RPM) at 37°C for 2 hours to simulate gastric emptying time (Chandler et al., 1997; Chandler et al., 1999; Sutton, 2004). Serial dilutions of the original inoculum and aliquots obtained from the simulated gastric incubations were plated and bacteria were counted to establish a pre- and post-quantification of aEPEC (CFU/ml).

**Clinical disease assessment.** Kittens were observed daily for clinical signs of illness including measurements of body weight, food consumption, and fecal consistency. Rectal temperature and heart rate were recorded every other day. Fecal consistency was scored using published criteria (Nestlé-Purina, St. Louis, Missouri; 63102) with scores ranging from 1 (very hard and dry) to 7 (watery with no texture). Fecal wet weight was determined by
obtaining an initial fecal weight followed by desiccation of feces at 60°C for 24 hours. Dried feces were weighed and the equation \( \frac{W_i - W_d}{W_i} \times 100 \) (\( W_i \) = initial fecal weight and \( W_d \) = dried fecal weight) was used to calculate the % fecal wet weight.

**Culture and molecular quantification of fecal aEPEC shedding.** Fecal culture of each kitten for detection of aEPEC shedding was performed every other day. Fecal material was diluted in sterile PBS and plated on MacConkey for isolation of Gram-negative bacteria. Eight distinct colonies were re-plated onto LB agar plates and biochemical tests including indole, pyrrolidonyl arylamidase, and oxidase (Beckton, Dickinson and Company, Franklin Lakes, NJ 07417) were used to identify the bacteria as *E. coli*. Each of the eight *E. coli* was frozen in 50% LB-glycerol freezing media and stored at -80°C. Each isolate was additionally collected using a 10 μl sterile loop and mixed with sterile nuclease free water for DNA extraction. DNA was liberated from bacteria by heating at 100°C for 30 minutes.

Identification of *E. coli* as EPEC was based on the amplification of *eae* using qPCR with previously published primers and reaction conditions (Franck et al., 1998). Three aEPEC isolates obtained from each kitten on day 3 post-infection were further characterized using pulsed-field gel electrophoresis. Molecular detection of aEPEC, by means of qPCR amplification of *eae*, was performed on DNA extracted from feces collected every other day prior to infection and daily for 7 days post-infection. DNA extractions were performed as previously described using a commercial kit (Zymo Research Corp. Irvine, CA) (Stauffer et al., 2008) and stored at -80°C prior to use. Quantitative PCR for *eae* was performed using previously published primers (Franck et al., 1998). Amplifications were performed with an
initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 45 seconds, and extension at 68°C for 1 minute.

**Pulsed-field gel electrophoresis.** Pulsed-field gel electrophoresis was performed as described for pulsenet analysis of *E. coli*, *Shigella*, and *Salmonella* (Ribot et al., 2006). Briefly, overnight cultures of EPEC were equilibrated in cell suspension buffer solution to an absorbency of 1.08-1.1 at 610 nm wavelength and mixed with 1% Seakem gold agarose (Thermo Fisher Scientific, Waltham, MA) for plug formation. The plugs were sectioned to an equal size with a 2-mm width and then individually digested with restriction enzyme *XbaI* (New England Biolabs, Ipswich, MA). Plugs were embedded into an agarose gel and restriction fragments were separated by electrophoresis at 6 volts/cm for 19 hours. Standardization across gels was confirmed by including *Salmonella enterica* serotype Braenderup H9812 digested with *XbaI* in each gel. Gels were stained with a commercially available DNA stain (GelRed, Biotium, Fremont, CA) following the manufacturer’s protocol and imaged with a UV imager (Bio-Rad, Hercules, CA). Gel images were subsequently inserted into and analyzed by BioNumerics software (Applied Maths, Inc., Austin, TX) as previously described (Swift et al., 2017).

**Intestinal function testing.** Intestinal function testing was performed on each kitten on day 6 of acclimation and again on day 4 post-infection with aEPEC. At each time period, kittens were lightly anesthetized by mask inhalation of isofluorane gas and then orogastrically dosed with a mixture of iohexol (Frias et al., 2012; Klenner et al., 2009) and D-xylose (Cabrera et al., 2013; Doerfler et al., 2000; Eberts et al., 1979; Merritt et al., 1986; Nix et al., 1993). Doses used for both iohexol and D-xylose were extrapolated from previously published
studies (Klenner et al., 2009; Nix et al., 1993). The final mixture contained 2 ml of a 350 mg/ml solution of iohexol (Omnipaque, GE Healthcare, Princeton NJ), 0.5 g of D-xylose (Sigma-Aldrich, Corp., St. Louis, MO), and sufficient sterile water to achieve a total solution volume of 11 ml. At 1.5 hours and 3 hours post-gavage, kittens were again lightly anesthetized by mask inhalation of isoflurane gas and a 1 ml whole blood sample was collected by jugular venipuncture. Blood samples were allowed to clot for 30 minutes and then centrifuged at 2,000 × g for 20 minutes to separate the serum from the red blood cells. Serum was stored at -20°C.

**D-xylose and iohexol assays.** Serum D-xylose concentrations were measured using a phloroglucinol assay as previously described (Doerfler et al., 2000; Eberts et al., 1979). The colorimetric reagent was composed of 0.25 g phloroglucinol (Sigma-Aldrich, Corp., St. Louis, MO), 50 ml of glacial acetic acid, and 5 ml HCl. Twenty μl of serum from each kitten was mixed with 2 mls of the color reagent and maintained at 100°C for 4 minutes and absorbency at 554 nm was measured using a spectrophotometer (BioTek Synergy 2, Biotek US, Winooski, VT). Standard dilutions of D-xylose in feline serum (Immunoreagents, Raleigh, NC) ranging from 3.125 mg/dl to 100 mg/dl were used to create a standard curve for each reaction. The direct impact of each experimental aEPEC isolate on D-xylose concentration was examined by co-culture of EPEC (10⁸ CFU each) in fresh LB containing D-xylose (5 mg/ml) at 37°C for 2 hours in triplicate. D-xylose remaining following incubation was measured using the phloroglucinol assay. A commercially available ELISA (BioPhysics Assay Laboratory Inc. Worcester, MA) was used to determine serum iohexol concentrations following the manufacturer protocol (Frias et al., 2012; Klenner et al., 2009).
Impact of oral administration of commensal feline Enterococcus hirae. For each experimental group of 8 kittens, individuals were randomly and evenly divided into two separate rooms containing 4 kittens each. Kittens in one room received a probiotic while those in the other received placebo/vehicle. The investigators were blinded as to which room of kittens received the vehicle or the probiotic. The administered probiotic was manufactured from a single, well-characterized isolate of Enterococcus hirae. The isolate (1002-2) was cultured from the mucosa-associated flora of the ileum from a healthy kitten in which histological evidence of E. hirae attachment to the intestinal epithelium was documented. The isolate lacked all virulence characteristics, the results of which were reported in another study (Ghosh et al., 2013).

The selected isolate of feline E. hirae was prepared for use as a probiotic by culturing in Biostat B-plus reactors fed with MB medium containing 20 g/L glucose using a variable speed pump. The dilution rate (D=0.17 h⁻¹), temperature (37°C), and pH (5.5) were maintained. The bacteria were collected from the reactor overflow and concentrated in PBS at pH=7. Numbers of viable cells were confirmed by plating on M17 agar and incubating at 37°C for 72 h. For each sample, three plates were prepared from each dilution and recorded as the average of three independent counts. Glucose consumption and accumulation of lactate and other fermentation products were monitored with high-performance liquid chromatography (Shimadzu Corporation, Kyoto, Japan) performed under isocratic conditions at 65°C, a mobile phase of 5 mM sulfuric acid (H2SO4) at a 0.4 ml/min flow rate using an Alltech IOA-1000 organic acids column (300 mm x 7.8 mm, Alltech, IL, USA), and coupled
to a refractive-index detector. Finalized probiotic and vehicle (bacteriological peptone) were stored at -80°C.

Aliquots containing 10-15 mg of probiotic powder (2.85 – 4.28 × 10⁸ CFU) or vehicle were prepared under sterile conditions on a single day and stored at -80°C until needed. One extra aliquot was prepared and plated to confirm CFU count. To confirm survival in a gastric environment, 2 additional aliquots were incubated in a simulated gastric environment as described for aEPEC. On each morning of administration, the aliquots were thawed, mixed with 100 μl of sterile water, and inoculated into ¼ teaspoon of canned cat food. The other 4 kittens per group received the vehicle prepared following the same methods used for the probiotic. The timing of administration of \textit{E. hirae} or vehicle in relation to other interventions in the study of each experimental group is shown in Figure 1.

**Confirmation of \textit{E. hirae} colonization.** Fecal culture for isolation of enterococci was performed every other day throughout the duration of the experiment. Fecal material was mixed with sterile PBS and plated on m-Enterococcus (BD Difco™, Fisher Scientific, Waltham MA) plates. Five individual isolates of enterococci were subcultured and each isolate was frozen in 50% LB-glycerol and stored at -80°C. Each isolate was additionally collected using a 10 μl sterile loop and mixed into sterile nuclease free water for DNA extraction. DNA was liberated from bacteria by heating at 100°C for 30 minutes. Enterococci were identified as \textit{E. hirae} by means of PCR using primers targeting the transglycosylase gene sequence specific to the species (\textit{murG}) (Arias et al., 2006). Quantitative PCR for \textit{murG} was performed to quantify the amount of \textit{E. hirae} DNA present on fecal DNA from each kitten. Amplifications were performed with an initial denaturation at 95°C for 2 minutes,
followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 2 minutes, and extension at 72°C for 3 minutes.

**Statistical analyses.** Data were tested for significant differences in distributions of observations between groups using chi-square and Fisher exact tests. Differences in the mean or median values of continuous data between groups of kittens were analyzed using Student’s t-tests or Mann-Whitney rank sum test, respectively. Changes in absorption of D-xylose, permeability to iohexol, and % fecal wet weight between baseline and post-infection measurements were analyzed by paired t-tests. Analyses were conducted using commercially available software (Sigmaplot 12, Systat Software, Inc., San Jose, CA) and significance was determined when P < 0.05.
Results

Establishment of infectious dose and simulated gastric survival of aEPEC. Using previously reported Ct values for *eae* amplified by qPCR from fecal DNA of 18 kittens that died or were euthanized due to diarrhea and comparing to a standard curve relating the Ct value of *eae* to CFU of EPEC in cat feces (Chapter II) it can be estimated that infected kittens shed a median of $1.8 \times 10^6$ (average $3.4 \times 10^7$) of aEPEC per 100 mg feces (range; $3.5 \times 10^3$ CFU to $2.0 \times 10^8$ CFU). The aEPEC isolates selected for use in experimental infections were documented to survive a 2-hour incubation in the simulated gastric environment (average CFU pre-incubation = $3.3 \times 10^7$, post-incubation = $2.95 \times 10^7$; averages determined from duplicate simulated gastric environments). Based on these findings, and infectious doses reported in other models of EPEC infection (Heczko et al., 2001; Savkovic et al., 2005; Splichalova and Splichal, 2012), an inoculation dose of $10^8$ CFU for each aEPEC isolate was selected for use in experimental infection.

Experimentally infected kittens shed live aEPEC. Prior to infection with aEPEC, 6/8 (75%) kittens in the first experimental group (no antibiotics) were discovered to be already carrying aEPEC on the basis of positive results of fecal culture (4 kittens), qPCR amplification of *eae* from fecal DNA (6 kittens), or both methods (4 kittens). In the second experimental group (antibiotics), kittens were pre-screened to confirm absence of aEPEC or *eae* in feces prior to purchase from the vendor. Despite this, 2/8 (25%) kittens were confirmed to be culture positive for aEPEC on arrival.

Following experimental infection, all kittens shed aEPEC, as determined both by isolation of aEPEC from feces and by direct qPCR amplification of *eae* from fecal DNA.
Using the cycle threshold values for \( eae \) to estimate CFU EPEC, an increase in shedding of aEPEC was documented in both groups of kittens (Figure 2). After determining the effects of aEPEC on kittens, aEPEC was eradicated from kittens using pradofloxacin (7.5 mg/kg once daily) (Bayer, Shawnee Mission, Kansas).

**Kittens shed only one of the pulsotypes of aEPEC administered for experimental infection.** While 3 isolates of aEPEC were administered to each kitten for induction of experimental infection, only 1 pulsotype (isolate 53-1) was subsequently shed (Figure 3). Among the 6 kittens in experimental group 1 (no antibiotics) that were found to be harboring their own aEPEC prior to infection, 2 shed only their native pulsotype after the experimental infection, 2 shed both their native pulsotype as well as isolate 53-1, and 2 shed only isolate 53-1. Despite the carriage of a native aEPEC at the time of purchase of 2 kittens in experimental group 2 (antibiotics), all kittens were treated with antibiotics prior to experimental infection after which only the dominant experimental isolate 53-1 was shed.

**Kittens with unaltered intestinal microbiota remain clinically asymptomatic carriers after experimental infection with aEPEC.** In the first experimental group of kittens that did not receive any pre-treatment with antibiotics, infection with aEPEC did not result in any significant changes in food intake, gain in body weight, rectal temperature, or heart rate (Figure 4 A and B). No significant changes in fecal scores or determinations of % fecal wet weight were observed as a result of aEPEC infection (Figure 4 C).

**Experimental infection of kittens with aEPEC results in significant occult changes in intestinal function.** Despite absence of clinical signs of diarrhea, aEPEC infection resulted in a significant decrease in intestinal absorption of orally-administered D-xylose (Figure 5
A). Importantly, *in vitro* culture of aEPEC with D-xylose (5 mg/ml) for a comparable (to *in vivo*) duration of 2 hours did not result in a decrease in D-xylose concentrations (post-aEPEC incubation D-xylose, 4.7 and 4.8 mg/ml). In addition to a decrease in intestinal absorption of D-xylose, aEPEC infection resulted in a significant decrease in intestinal permeability to orally-administered iohexol (Figure 5 B).

**Kittens with antibiotic-altered intestinal microbiota become susceptible to aEPEC infection-associated changes in fecal consistency.** While receiving antibiotics, there was a decrease in bacterial growth on m-Enterococcus plates and no Gram-negative bacteria were cultured. Similar to the group of kittens that did not receive antibiotics prior to infection with aEPEC, kittens that were pre-treated with antibiotics demonstrated no effect of aEPEC infection on food intake, gain in body weight, rectal temperature, or heart rate (Figure 6 A and B). During treatment with antibiotics, however, significant increases were observed in fecal scores and % fecal wet weights that were presumably reflective of intended disruption of the intestinal microbiota. These values returned to normal over the 72 hour “antibiotic washout” period prior to experimental aEPEC infection (Figure 6 C). After introduction of aEPEC infection, recurrent significant increases were observed in both fecal score and % fecal wet weight (Figure 6C). Compared to baseline, kittens that were pre-treated with antibiotics had decreases in post-aEPEC infection absorption of D-xylose and permeability to iohexol, however these changes were not statistically significant (Figure 7).

**Oral administration of commensal *E. hirae* results in increased fecal shedding based on molecular detection and culture.** To determine the impact of oral supplementation with a commensal member of the mucosa-associated microbiota on manifestations of experimental
aEPEC infection, kittens in both experimental groups were randomized to receive either a probiotic formulation of *E. hirae* or vehicle. Using a species-specific qPCR assay for the detection of *E. hirae murG*, all kittens were demonstrated to carry *E. hirae* as part of their normal flora. As demonstrated in the group of kittens that did not receive pre-treatment with antibiotics, oral administration of *E. hirae* was accompanied by significant increases in the quantity of fecal *murG* as compared to kittens that received only the vehicle (Figure 8). *Enterococcus hirae* were only cultured from kittens that received the probiotic in this study; 60% (12/20) of the enterococcus isolates were confirmed as *E. hirae*. There was a large variability in *murG* quantity and quantity of cultured *E. hirae* in the kittens that received antibiotics prior to probiotic administration. No adverse health effects such as changes in food intake, body weight, rectal temperature, or heart rate were observed in kittens administered *E. hirae* compared to those that received vehicle.

**Probiotic composed of *E. hirae* can promote aEPEC associated decrease in intestinal permeability independent of changes in burden of aEPEC infection.** In kittens that did not receive pre-treatment with antibiotics, *E. hirae* or vehicle was administered daily for 5 days prior to infection with aEPEC (Figure 1 A). The kittens that received *E. hirae* prior to infection with aEPEC had a significantly greater decrease in intestinal permeability to iohexol compared to those receiving vehicle (Figure 5B). In kittens that received antibiotics prior to infection with aEPEC, *E. hirae* or vehicle was administered daily for 2 days prior to infection (during “antibiotic washout”) and daily thereafter throughout the infection (Figure 1B). The kittens that received *E. hirae* had a non-significantly lower fecal wet weight (mean=67%) on day 3 post-infection when compared to kittens receiving the vehicle.
Additionally, kittens that received *E. hirae* had significantly lower permeability to iohexol post-infection compared to the kittens that received vehicle (*P*<0.05; Student’s T-test) (Figure 7B). There was no difference in quantity of EPEC cultured from the feces of kittens that received the probiotic compared to those receiving vehicle in either group of kittens (Table 1). No significant differences in absorption of D-xylose were observed on the basis of *E. hirae* or vehicle administration in either group of kittens (Figure 5A and 7A).
Discussion

This study demonstrates the effects of aEPEC infection on experimentally infected kittens with and without antibiotic-induced alterations of the gut microbiota. We established that a kitten derived aEPEC is able to survive the gastrointestinal tract leading to fecal shedding by experimentally infected kittens. Kittens in this study had limited clinically evident signs of disease (i.e. no diarrhea). However, there were subclinical alterations to intestinal absorption as measured by D-xylose. Although not recognized clinically as diarrhea, kittens with antibiotic-induced microbiota alteration demonstrated a significant increase in fecal score and fecal wet weight at 3 days post-infection.

Kittens, at 4 days-post-infection with EPEC, had significantly decreased absorption of D-xylose, indicating an intestinal malabsorption. Infection with EPEC could induce malabsorption in at least 3 ways: 1) by adhering to microvilli, impacting actin and cytoskeleton leading to loss of microvilli and thus loss of absorptive surface area (Moon et al., 1983); 2) causing overall villus blunting and loss of surface area (Fagundes-Neto et al., 1997; Ulshen and Rollo, 1980); and 3) directly inhibiting the transport protein sodium-glucose cotransporter (SGLT-1) (Dean et al., 2006). The malabsorption present post-infection in these kittens was likely due to a combination of these EPEC-associated disease mechanisms. In addition to the direct effect of EPEC infection on D-xylose absorption, EPEC infection could have induced a change in the intestinal microbial community, leading to changes in bacteria that metabolize D-xylose and changes in D-xylose available for the host to absorb. Likewise, changes could have occurred in bacteria with the ability to hydrolyze xylan to produce xylose (Ivarsson et al., 2014). Kittens with an antibiotic-induced
altered gut microbial community also had a decrease in D-xylose absorption, but the low starting values perturbed significance. Lastly, administration of the probiotic had no significant impact on the malabsorption identified as associated with EPEC infection.

In contrast to _ex vivo_ and _in vitro_ data demonstrating that EPEC causes increased paracellular permeability, our _in vivo_ data utilizing iohexol indicated a decrease in permeability in kittens that did not receive antibiotics prior to infection. We hypothesized that two EPEC-induced mechanisms could lead to decreased permeability. One possible mechanism is that EPEC induced prostaglandin release from the epithelial cells lining the intestine. Prostaglandin has been shown to be associated with decreased permeability due to enhanced barrier function with no change in mucosal surface area (Gookin et al., 2003). Another possible cause for the decrease in permeability is villus blunting. Children with EPEC often have villus blunting (Fagundes-Neto et al., 1997; Rothbaum et al., 1982; Ulshen and Rollo, 1980) which decreases overall surface area. Decreased surface area could lead to decreased paracellular spaces and overall decreased areas for permeability. On the other hand, our study indicates that the decrease in permeability was associated with the use of the probiotic. Lastly, the kittens that did not receive antibiotics prior to infection had a higher baseline permeability as indicated by higher serum concentrations of iohexol at baseline.

In addition to demonstrating the effects of EPEC experimental infection on kittens, this study is the first to analyze the use of _E. hirae_ as a probiotic in cats. Kittens receiving the _E. hirae_ probiotic post-antibiotic induced microbial disruption had lower, although not significantly, fecal wet weights at 3 days post-infection with EPEC. The lack of significance may indicate that this difference is not clinically relevant. However at 4 days post-infection,
kittens receiving the probiotic had a lower average iohexol serum concentration, indicating decreased permeability could be associated with the difference in fecal wet weight. Likewise, the kittens that did not receive antibiotics but received probiotics prior to infection had a significantly higher change in permeability to iohexol between baseline and post-infection, indicating a decrease in permeability and stronger barrier. This preliminary study indicates that *E. hirae* may act as a protective, beneficial probiotic in kittens at risk for enteroadherent bacterial infection or other intestinal diseases impacting barrier function and permeability.

Previous studies on the probiotic benefits of *E. hirae* have been performed. *In vitro* studies demonstrated that an isolated *E. hirae* from the rumen of an Aurochs bovine was adherent to Caco-2 cells, limited inflammatory response to LPS, and was antimicrobial against all assayed enteric bacteria (Arokiyaraj et al., 2014). In our lab we have shown that *E. hirae* is adherent to intestinal porcine epithelial cells from the small intestine (IPEC-J2 cells) and our previous study also demonstrated the ability of *E. hirae* to adhere to intestinal epithelial cells *in vivo* in kittens (Ghosh et al., 2013). In another *in vitro* study, the lipoteichoic acid (LTA) component of the cell wall of a commercially available strain of *E. hirae* (ATCC 9790) was able to increase trans-epithelial electrical resistance post TNFα-induced injury to barrier function via enhancing the expression of tight junction proteins (Miyauchi et al., 2008). As has been demonstrated by *E. hirae*, other members of the gut microbiota have similarly induced enhanced barrier function *in vivo* and *in vitro* which is often modulated by increased expression of tight junction proteins (Dicksved et al., 2012; Hsieh et al., 2015; Miyauchi et al., 2009; Miyauchi et al., 2012; Yang et al., 2015).
ability of resident microbes to maintain the intestinal barrier represents an excellent beneficial mechanism of a potential probiotic, like *E. hirae*.

This study included some limitations which are worth mentioning. First, due to our interest in confirming disease causation for EPEC and the potential protective benefits of our probiotic, we limited the kittens to positive controls (infected) and treated controls. Therefore, we did not include negative controls and were unable to perform the intestinal function assays on age-matched negative controls. However, it is highly unlikely that changes noted in D-xylose absorption and iohexol permeability were age/growth associated as all kittens were weaned at both time-points, the assays were performed within 10 days of each other, and no significant differences were present when kittens were grouped by weight. Another limitation is that intestinal tissues were not examined to confirm colonization and enteroadherence of EPEC. We, purposefully, did not obtain tissues post-mortem because this was a non-terminal study and biopsies were not performed to minimize pain, distress, and injury to the kittens. Lastly, we were unable to obtain EPEC negative kittens from the commercial vendor, indicating a high prevalence of EPEC in kittens from this vendor. However, comparisons of data from kittens that arrived with a naturally-acquired EPEC and those not infected with EPEC did not elucidate any significant differences in response to probiotic or EPEC infection. The retention of the naturally acquired aEPEC in kittens in the first study (no antibiotics) may indicate that previous infection with aEPEC may protect against infection with a different isolate of aEPEC.

This study demonstrated that aEPEC alters intestinal absorption in kittens. As demonstrated by evaluations of fecal consistency, there is likely an association between
intestinal microbial disruption and increased impact of aEPEC infection. Furthermore, the *E. hirae* probiotic may decrease permeability in kittens, indicating an improved intestinal barrier function. However, the *E. hirae* probiotic did not impact aEPEC infection in these kittens. A probiotic composed of *E. hirae* represents a possible therapeutic in kittens at risk for increased permeability, but could also be of benefit to children, particularly children at risk for EPEC infection.
### Tables

Table 1. Comparison of cultured EPEC form kittens that received or did not receive probiotics.

<table>
<thead>
<tr>
<th>Study group 1 (no antibiotics)</th>
<th>No. (%) <em>E. coli</em> identified as EPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr PI (n=32)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Probiotic</td>
<td>26 (81)</td>
</tr>
<tr>
<td>No probiotic</td>
<td>26 (81)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study group 2 (antibiotics)</th>
<th>No. (%) <em>E. coli</em> identified as EPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr PI</td>
</tr>
<tr>
<td>Probiotic</td>
<td>32 (100)</td>
</tr>
<tr>
<td>No probiotic</td>
<td>32 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Eight *E. coli* selected from each kitten, yielding a total of 32 from each group (either probiotic treated or not) of kittens.
Figure 1. Experimental timeline for studies. The timelines for kittens that did not receive antibiotics prior to infection (Figure 1 A) and kittens that received antibiotics prior to infection (Figure 1 B) are included. The arrows indicate the day of intestinal function assays. WO = wash out of antibiotics from intestinal tract.
Figure 2. Confirmation of aEPEC infection in kittens. Detection of EPEC was performed using qPCR for amplification of *eae* in fecal DNA. The Ct values were used to calculate the CFU of aEPEC based on the standard curve demonstrated in Chapter II. Panel A demonstrates the estimated CFU of aEPEC in kittens that did not receive antibiotics prior to infection. Three kittens did not amplify *eae* and therefore had no EPEC CFU detected. Panel B demonstrates the CFU of aEPEC in kittens that did receive antibiotics prior to infection. On the day prior to infection, no *eae* was amplified from fecal DNA from the kittens that received antibiotics (represented by data-points on x-axis). Black circles = kittens that received probiotic; white circles = kittens that received vehicle only.
Figure 3. Designation, serotype, and pulse-field gel electrophoresis pulsotype of the 3 isolates used for experimental infection and 2 isolates carried by kittens at the time of purchase by a commercial vendor.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Serotype</th>
<th>Pulsotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-1</td>
<td>O108:H21</td>
<td></td>
</tr>
<tr>
<td>53-1</td>
<td>O153:H21</td>
<td></td>
</tr>
<tr>
<td>33-2</td>
<td>O111:H8</td>
<td></td>
</tr>
<tr>
<td>Vendor-1</td>
<td>O88:H-</td>
<td></td>
</tr>
<tr>
<td>Vendor-2</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Kittens that did not receive antibiotics prior to infection remained clinically healthy throughout the course of the experiment. Panel A demonstrates the average food intake (g) (black circles) and average body weight (kg) (grey circles) of the kittens. Panel B demonstrates the average rectal temperature of the kittens. Panel C demonstrates the average fecal wet weights (%) (black circles) and median fecal scores (grey circles). Missing data-points were removed from food intake graph as food was removed for the duration of the intestinal function assay (9-10 hours). Data-points were similarly removed from fecal consistency graph as intestinal function assays influenced fecal consistency. Each experimental time-course is demonstrated along the x-axis and the vertical line indicates day of infection. Acc=acclimation; Pro=probiotic; Inf=infection; Erad=eradication
Figure 5. Intestinal function assays to determine the impact of EPEC infection or probiotic administration to kittens with no antibiotic-induced alteration of the microbiota. Panel A shows the serum D-xylose concentrations 1.5 hours post-administration to kittens, at baseline (acclimation day 6) and 4-days-post-infection. The average serum D-xylose concentration was significantly lower at 4 days-post-infection (*P<0.05, paired T-test). In panel B, the average serum iohexol concentrations obtained 1.5 hours post-administration are shown for comparison between baseline and 4-days-post-infection. The average serum iohexol concentration was significantly lower at 4-days-post-infection (**P<0.01, paired T-test). The black circles represent kittens that received the probiotic and the white circles represent the kittens that received vehicle only. The horizontal lines mark the average value at each time-point.
Figure 6. Kittens that received antibiotics prior to infection remained clinically healthy but had showed significant increases in their fecal consistency during antibiotic administration and 3 days post-infection. Panel A demonstrates the average food intake (g) (black circles) and average body weight (kg) (grey circles) of the kittens. Panel B demonstrates the average rectal temperatures of the kittens. Panel C demonstrates the average fecal wet weights (%) (black circles) and median fecal scores (grey circles) (*P<0.05; paired T-test comparing to % fecal wet weights on day 5 of acclimation and Mann-Whitney Rank Sum test comparing to fecal scores on day 5 of acclimation). Missing data-points were removed from food intake graph as food was removed for the duration of the intestinal function assay (9-10 hours). Data-points were similarly removed from fecal consistency graph as intestinal function assays influenced fecal consistency. Each experimental time-course is demonstrated beneath the graph. Acc=acclimation; Pro=probiotic; Inf=infection; Erad=eradication
Figure 7. Intestinal function assays to determine the impact of EPEC infection in kittens with antibiotic-induced alteration of the microbiota. Panel A shows the serum D-xylose concentrations 1.5 hours post-administration to kittens, at baseline (acclimation day 6) and 4-days-post-infection. The average serum D-xylose concentration was lower at 4 days-post-infection; however, the difference was not significant. In panel B, the average serum iohexol concentrations obtained 1.5 hours post-administration are shown for comparison between baseline and 4-days-post-infection. There was no significant change in the average serum iohexol concentration. The y-axis scale for iohexol permeability reflects the lower baseline permeability in this group of kittens. The black circles represent kittens that received the probiotic and the white circles represent the kittens that received vehicle only. The horizontal lines mark the average value at each time-point.
Figure 8. Effect of the probiotic composed of *E. hirae* on the shedding of *E. hirae* in kittens that did not receive antibiotics prior to probiotic administration (first study). Using qPCR, we demonstrated an increase in the presence of the *E. hirae* specific gene (*murG*) in the fecal DNA of kittens that received the probiotic. The black circles represent kittens that received probiotic and the white circles represent kittens that received vehicle. Acc=acclimation; Pro=probiotic; Inf=infection; Erad=eradication
CHAPTER V

Conclusions and Future Directions

Enteropathogenic *E. coli* remains an important cause of diarrhea in children. Even though it is often not considered one of the top 5 agents of diarrhea, tEPEC and undifferentiated EPEC (not determined as aEPEC or tEPEC) are surprisingly associated with increased risk of mortality (Kirk et al., 2015; Kotloff et al., 2013). We determined that kittens can be colonized by aEPEC and the prevalence of aEPEC in kittens was 17%. We have demonstrated an association of diarrhea-related mortality and EPEC in kittens in the U.S. Furthermore, we discovered a significant association between the presence of EPEC and the need for parenteral fluids. This likely indicates an association of EPEC with dehydration, which has similarly been shown in humans infected with EPEC (Dutta et al., 2013) and has been demonstrated in a mouse model (Guttman et al., 2007). By studying naturally infected kittens, we have also demonstrated that inflammation in the lamina propria of the small intestine and colon is associated with aEPEC. Furthermore, aEPEC isolated from kittens are genomically similar to EPEC isolated from children. Overall these studies demonstrate that kittens may represent a naturally occurring animal model to study *in vivo* aEPEC pathogenesis and may act as a reservoir for zoonotic transmission of aEPEC to children.

Elucidating the *in vivo* pathogenesis of EPEC in kittens can also lead to discovery of novel therapeutics and treatments to ameliorate diarrheal disease in both children and kittens. In our preliminary study of experimental infection of kittens with EPEC, we aimed to determine the effects of EPEC on intestinal function, the impact of pre-infection gut microbial alteration (“dysbiosis”) on EPEC infection, and to investigate the ability of a novel
probiotic, composed of *Enterococcus hirae*, to protect against the deleterious effects of EPEC. We showed that EPEC infection leads to malabsorption as determined by the absorption of orally administered D-xylose. Kittens administered broad-spectrum antibiotics prior to EPEC infection had increased fecal scores and fecal % wet weights at 3 days-post-infection. This increase in fecal consistency indicating more water loss indicates that a pre-infection “dysbiosis” accentuates disease due to EPEC. Although we did not determine an effect of the probiotic on EPEC infection, we did show administration of the probiotic was associated with a decrease in intestinal permeability as determined by orally administered iohexol and subsequent serum concentration. The serum concentration of iohexol in kittens that received the probiotic was lower than kittens that did not receive the probiotic. These data indicate that *E. hirae* leads to an enhanced barrier function, which is likely due to increased expression of tight junction protein, zonula occludens-1, as demonstrated in a previous study (Arokiyaraj et al., 2014; Miyauchi et al., 2008). However, our study was unable to determine the mechanism of decreased permeability in kittens. Probiotics are increasingly investigated for their benefit to hosts with inflammatory bowel disease and infectious diseases. This preliminary study investigated the potential use of a probiotic composed of *E. hirae* in kittens with EPEC; further work will focus on the ability of *E. hirae* to enhance barrier function in the presence and absence of EPEC.

Additional future work will focus predominantly on the role of the microbiome in protecting the host from infection and the structure of the microbiome in animals with aEPEC and diarrhea compared to animals with aEPEC and no diarrhea. It has been established that animals and children can be infected with aEPEC but never develop clinical
signs. Therefore, it is highly likely that the microbiome differs in these populations. In addition to determining differences in microbiome diversity and populations, future work will also turn to the metabolome to determine the functions of the members of the microbiome in either population. Investigation of the microbiome and metabolome in kittens, other naturally infected animal models, and children should elucidate a particular pattern that may or may not be shared across species that protects the host from EPEC infection or a particular pattern that increases susceptibility to EPEC infection.

Overall, these studies augment the current understanding of aEPEC in the feline model, elucidate associations of aEPEC infection and disease in kittens, and demonstrate a potential beneficial probiotic to enhance barrier function of the intestine. Previous studies have postulated that cats are a natural reservoir for EPEC and these studies have likewise shown that kittens shed EPEC that are genomically similar to EPEC isolated from children in developing countries. EPEC strains were easily transmitted and shared among the kittens in the experimental study and transmission between a puppy and child has been shown previously (Rodrigues et al., 2004a). Therefore, it is likely that EPEC can be transmitted from kittens to children; however, EPEC is not commonly identified in children in the US. Companion animals in developing countries, where EPEC is more prevalent, likely carry and shed even higher amounts of EPEC and additional differences in the microbiome or innate immune defense in children allows for enhanced disease.
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