Enteropathogenic *Escherichia coli* (EPEC) expressing a non-functional bundle-forming pili (BFP) also leads to increased growth failure and intestinal inflammation in C57BL/6 mice

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**Abstract**

Bundle-forming pili (BFP) are implicated in the virulence of typical enteropathogenic *E. coli* (EPEC), resulting in enhanced colonization and mild to severe disease outcomes; hence, non-functional BFP may have a major influence on disease outcomes in vivo. Weaned antibiotic pre-treated C57BL/6 mice were orally infected with EPEC strain UMD901 (E2348/69 *bfpA* C129S); mice were monitored daily for body weight; stool specimens were collected daily; and intestinal tissues were collected at the termination of the experiment on day 3 post-infection. Real-time PCR was used to quantify fecal shedding and tissue burden. Intestinal inflammatory biomarkers lipocalin-2 (LCN-2) and myeloperoxidase (MPO) were also assessed. Infection caused substantial body weight loss, bloody diarrhea, and intestinal colonization with fecal and intestinal tissue inflammatory biomarkers that were comparable to those previously published with the wild-type typical EPEC strain. Here we further report on the evaluation of an EPEC infection model, showing how disruption of *bfp* function does not impair, and may even worsen diarrhea, colonization, and intestinal disruption and inflammation. More research is needed to understand the role of *bfp* in pathogenicity of EPEC infections in vivo.

**Keywords** Enteropathogenic *E. coli* · Bundle-forming pili · Murine model · Diarrhea · Inflammation

**Introduction**

Diarrhea continues to be a problem in young children especially those infected with enteropathogenic *E. coli* (EPEC) [1, 2]. The classification of enterohemorrhagic *E. coli*
(EHEC) and EPEC (typical and atypical strains) pathotypes has resulted in a significant increase in knowledge of the epidemiology, pathophysiology, and clinical presentation of these pathogen infections [3–5]. Aside from their common virulence determinants, these pathotypes differ in their virulence depending on the presence or absence of bundle-forming pili (BFP), the EPEC adherence factor (EAF) plasmid, and Shiga toxin production [5–7]. EPEC causes moderate to severe diarrhea in children under 12 months of age [2]. Typical EPEC strains demonstrate distinct localized adherence in cell lines and in tissue biopsies [8, 9]. This distinct pattern is associated with protruding flagella and BFP which are thought to assist in contact to the host cells, followed by intimate adherence resulting in attaching and effacing lesions with actin accumulation at the site of infection [10, 11].

BFP is a type IV fimbriae [12] encoded by the EAF plasmid [13, 14]. It consists of a cluster of 14 genes, with the bfpA gene encoding the major adhesin subunit (bundlin) and the 13 other genes (bfpB to bfpL) involved in BFP biogenesis [15–18]. BFP is produced during bacteria-bacteria interaction resulting in a mesh-work of fibers that lead to microcolony formation, enhancing the stability of EPEC on the infected intestinal mucosa [11, 17, 19]. The EAF plasmid also consists of the plasmid-encoded regulator (per) locus [20] and it has 99% sequence similar to BFP; they have both been reported to cause increased disease outcome in human volunteers [10, 15].

Atypical EPEC lacks the EAF plasmid and, therefore, cannot produce BFP [21]. Atypical EPEC strains are distinguished by their different adherence patterns on cultured epithelial cells. They are becoming increasingly recognized in clinical settings, both in symptomatic and asymptomatic individuals [2, 10], and have also been reported to cause prolonged diarrhea in children [22, 23]. Atypical strains are highly diverse and may cause damage to the host through a variety of ways [10]; therefore, studying the effects of the lack of bfp in EPEC infection is important in understanding the disease outcomes. We have recently described an EPEC infection model using weaned mice and showed that EPEC prototype strain E2348/69 was able to colonize the intestine and caused weight decrements, overt diarrhea, intestinal disruption, inflammatory responses, and systemic metabolic perturbations [24]. In this follow-up report, we studied the effects of the prototype EPEC strain (E2348/69) that carries a mutated bfpA (site-directed mutant strain UMD901) in the same model to determine whether a non-functional BFP in EPEC leads to altered disease outcomes and compare the results to those of typical EPEC (functional BFP) previously reported [24].

Methods

Animal husbandry

Male, 22-day-old C57BL/6 mice utilized in this study were obtained from Jackson Laboratories (Bar Harbor, USA). Upon arrival, mice weighed about 10 g and were co-housed in groups of up to 4 animals per cage. The temperature of the vivarium was regulated between 20 and 23 °C, operating at a 10-h dark and 14-h light cycle. Upon their arrival, mice were given 3 days to acclimate and were placed on standard rodent house chow diet (Harlan), prior and after the infection. Four days before the infection challenge, mice were given an antibiotic cocktail added to the drinking water for 3 days. Mice were then given clean drinking regular water without antibiotics for 24 h as previously described [25]. Throughout the infection period, no antibiotic cocktail was administered. All procedures were equally followed for both infected and uninfected animals.

EPEC mouse challenge

UMD901 [26] was cultivated in 20 mL Dulbecco’s modified Eagle’s medium (DMEM) containing phenol red and incubated at 37 °C in a shaking incubator until optimal growth of OD600 ~ 0.6 was achieved. Cultures were centrifuged at 3500 × g for 10 min at 4 °C, and the pellet was resuspended in DMEM high glucose to obtain 1010 CFU/mL. Using 22-gauge feeding needles, mice were inoculated with 100 μL of this bacterial suspension by oral gavage. Uninfected mice were gavaged with 100 μL of DMEM high glucose as control.

Throughout the 3-day infection period, all mice were weighed and stools collected daily. After this period, euthanasia of the animals was performed. Intestinal tissues (duodenum, jejunum, ileum, and colon) and cecal contents were collected, flash frozen in liquid nitrogen and kept at –80 °C until further investigation.

Stool shedding and tissue burden

DNA was extracted from fresh stool specimens collected at days 1 and 3 post-infection (p.i.) using the QIAamp DNA stool mini kit (Qiagen). DNA from intestinal tissues was extracted using the DNeasy Kit (Qiagen). The eae gene with the primer sequences: 5’-CCCCAACATCGGC ACAAGCATAAGC-3’ (sense) and 5’-CCCCGATCCGTG TCGCCAGTATTCCG-3’ (antisense) [27] was used as the target. Stool shedding and tissue burden were quantified using real-time PCR with the following conditions: 95 °C
for 3 min, then 40 cycles for 15 s at 95 °C, 60 s at 55 °C, and 72 °C for 20 s.

**Intestinal inflammatory response**

Protein lysates from the stools and cecal contents were extracted using radioimmuno-precipitation assay buffer [25]. After centrifuging the lysates at 8000 × g for 5 min, the supernatant was utilized to perform the protein assay using the bicinchoninic acid assay (Thermo Fisher Scientific, USA). Inflammatory biomarkers lipocalin-2 (LCN-2) and myeloperoxidase (MPO) were measured using a commercial ELISA kits (R&D Systems, USA).

**Statistical analysis**

Data presented in the study was analyzed using GraphPad prism 8 (GraphPad Inc., USA). All statistical analyses were performed on raw data using two-way ANOVA and Turkey’s post hoc and the Kruskal–Wallis and Dunn’s multiple comparison tests where applicable. Data presented are the mean and standard error of the mean (SEM); differences were considered significant with *p*-value of < 0.05.

**Results**

**Non-functional BFP leads to weight loss, bloody stools, and colonization**

C57BL/6 mice pre-treated with antibiotic cocktail 3 days prior to infection were infected with strain UMD901 (E2348/69 *bfpA C129S*) and monitored daily for changes in weight. The same procedure was performed in parallel with wild-type (WT) EPEC E2348/69 strain, the results of which have been already published elsewhere [24]. For comparison purposes, some of the results already published for this strain have been extracted and included to this report. A significant weight loss of mice infected with strain UMD901 was observed at days 1 and 2 p.i. when compared to uninfected (*p* = 0.003) and WT-infected mice (*p* = 0.003) (Fig. 1A). UMD901-infected mice also had a significant weight change at day 3 p.i. when compared to the uninfected mice (*p* = 0.0004). The effect of strain UMD901 infection in C57BL/6 mice was also assessed based on the consistency and appearance of stools (Fig. 1B). All uninfected mice had well-formed stools. In contrast, 8 of 12 mice infected with strain UMD901 developed bloody stools at days 1 and 2 p.i. and at day 3 p.i. had diarrhea characterized by wet bottom and liquid stools. WT-infected mice also had wet stools, but
with no blood. During the infection period, stool specimens were collected and quantified for EPEC shedding using quantitative PCR (Fig. 1C). WT shedding was observed with a mean of $2.3 \times 10^9$ organisms/10 mg stool while UMD901 shedding had a mean of $4.5 \times 10^7$ organisms/10 mg stool at day 1 p.i.; however, no statistically significant difference was observed. In order to evaluate strain UMD901 colonization, intestinal tissues from the duodenum, jejunum, ileum, and colon were collected and analyzed for tissue burden using PCR following a 3-day infection period (Fig. 1D). We have previously shown that WT EPEC (E2348/69) colonizes across all tissue sections at day 3 p.i. with the highest tissue burden observed in the ileum and colon [24] (data included in Fig. 1D). In this study, strain UMD901 was found to also colonize all tissue sections, with higher tissue burden being observed in the colon.

**Infection with WT and UMD901 strains leads to increased inflammation**

An increase in neutrophil infiltration as a result of intestinal infection is associated with increased biomarkers such as MPO and LCN-2 [28]. Stool specimens collected at day 2 p.i. were analyzed for MPO and LCN-2 inflammatory biomarkers (Fig. 2A). MPO levels of mice infected with WT [24] and UMD901 strains were significantly higher when compared to uninfected mice ($p=0.003$, $p=0.0009$) respectively. A significant difference of fecal inflammatory LCN-2 was observed in mice infected with strain UMD901 when compared to the uninfected group ($p=0.03$) at day 2 p.i. Following euthanasia at day 3 p.i., cecal contents were collected and evaluated for MPO and LCN-2 biomarkers. As seen in Fig. 2B, increased levels of MPO and LCN-2 were also observed to be higher in mice infected with WT and UMD901 strains, when compared to uninfected mice ($p=0.03$).

**Discussion**

The role of EPEC during infection has been reported [14, 29, 30]. Recently, we have developed a murine EPEC infection model using weaned C57BL/6 mice that have been pre-treated with antibiotic cocktail, demonstrating weight alterations, overt diarrhea, intestinal inflammation, and metabolic perturbations [24]. This model also demonstrated that WT EPEC (E2348/69) infection causes watery diarrhea, an important feature that has been reported in humans. Based on the previous EPEC murine model findings, we further evaluated the influence of BFP, which is one of the main traits thought to be involved in typical EPEC localized adherence, microcolony formation, antigenicity, and...
Various diarrheagenic *E. coli* pathotypes [36, 37]. Atypical EPEC O26 strains have been suggested to be descendants of EHEC O26 that may have lost the Shiga toxin; yet, this EPEC strain has also been linked to bloody diarrhea [33, 38]. Ruiz et al. [37] also reported on an uncommon atypical EPEC strain expressing a plasmid-encoded toxin that has only been documented in EAEC to induce cell damage by altering the cytoskeletal structure. In this model, we therefore predict that the strain UMD901 colonized extensively in multiple intestinal regions, as demonstrated in all the intestinal tissue sections throughout the 3-day infection period, causing increased inflammation and bloody stools. Furthermore, these findings suggest that EPEC infection in mice does not need a functioning *bfpA* for enhanced virulence.

Although comparing animal studies to humans has limitations due to differences in microbiota, colonization of strain UMD901 in all intestinal regions implies that loss of BFP function does not affect broader colonization. These results are in accordance with a previous report by DuPont et al. [29] indicating that *bfpA* is not critical during colonization in neonatal mouse model when compared to WT EPEC. Based on these findings, this could potentially explain the high prevalence of atypical EPEC strains in clinical studies [23, 39, 40]. Bieber et al. [15] previously reported on *bfpA* infection with 2 out of 16 human volunteers developing mild diarrhea and humans infected with the WT EPEC B171-8 strain developing a more severe diarrhea. In conclusion, infection of EPEC without a functional BFP in weaned mice pre-treated with antibiotics causes bloody appearance in stools, accompanied with growth failure and colonization leading to increased inflammatory biomarkers. The potential of further studying atypical EPEC clinical strains in this model is therefore warranted in order to understand the mechanisms of pathogenesis.

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**Author contribution** S. E. L., D. T. B., A. N. T., N. P., R. L. G., and J. P. N. conceived and designed the experiment. S. E. L., D. T. B., P. H. Q. S. M., and G. L. K. participated in the experiment. S. E. L. and D. T. B. contributed to data analysis. S. E. L. wrote the manuscript draft. All authors read and approved the final manuscript.

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**Data availability** The datasets presented during the current study are available on reasonable request.
Declarations

Ethics approval The mice used in the study were maintained in strict compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals of the [41]. The protocol was accepted by the University of Virginia’s Committee on the Ethics of Animal Experiments (Protocol Number: 3315). Every attempt was made to reduce suffering. This study complies with the University of Virginia’s Institutional Animal Care and Use Committee policies. University of Virginia is recognized by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

Conflict of interest The authors declare no competing interests.

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