Higher atypical enteropathogenic 
*Escherichia coli* (a-EPEC) bacterial loads 
in children with diarrhea are associated 
with PCR detection of the EHEC factor 
for adherence 1/lymphocyte inhibitory factor A 
(*efa1/lifa*) gene

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**Abstract**

**Background:** Typical enteropathogenic *Escherichia coli* (t-EPEC) are known to cause diarrhea in children but it is uncertain whether atypical EPEC (a-EPEC) do, since a-EPEC lack the bundle-forming pilus (*bfp*) gene that encodes a key adherence factor in t-EPEC. In culture-based studies of a-EPEC, the presence of another adherence factor, called EHEC factor for adherence/lymphocyte activation inhibitor (*efa1/lifA*), was strongly associated with diarrhea. Since a-EPEC culture is not feasible in clinical laboratories, we designed an *efa1/lifA* quantitative PCR assay and examined whether the presence of *efa1/lifA* was associated with higher a-EPEC bacterial loads in pediatric diarrheal stool samples.

**Methods:** Fecal samples from children with diarrhea were tested by qPCR for EPEC (presence of *eae* gene) and for shiga toxin genes to exclude enterohemorrhagic *E. coli*, which also contain the *eae* gene. EPEC containing samples were then tested for the bundle-forming pilus gene found in t-EPEC and *efa1/lifA*. The *eae* gene quantity in *efa1/lifA*-positive and negative samples was compared.

**Results:** Thirty-nine of 320 (12%) fecal samples tested positive for EPEC and 38/39 (97%) contained a-EPEC. The *efa1/lifA* gene was detected in 16/38 (42%) a-EPEC samples. The median *eae* concentration for *efa1/lifA* positive samples was significantly higher than for *efa1/lifA* negative samples (median 16,745 vs. 1183 copies/µL, respectively, p = 0.006).

**Conclusions:** Atypical enteropathogenic *E. coli*-positive diarrheal stool samples containing the *efa1/lifA* gene had significantly higher bacterial loads than samples lacking this gene. This supports the idea that *efa1/lifA* contributes to diarrheal pathogenesis and suggests that, in EPEC-positive samples, *efa1/lifA* may be a useful additional molecular biomarker.

**Keywords:** Enteropathogenic *Escherichia coli*, Diarrhea, Real-time PCR, Gastroenteritis
Background

Enteropathogenic *Escherichia coli* (EPEC) is not detected with the standard stool culture methods used in clinical laboratories for bacterial pathogens, so its relative importance as a cause of diarrhea in children has been uncertain in the past [1]. With the recent increased use of molecular detection methods, however, EPEC has been found to be the most frequently detected bacterium in patients with diarrhea in developed countries [2, 3].

However, not all EPEC strains have the same ability to cause diarrhea. All EPEC and enterohemorrhagic *E. coli* (EHEC) contain the chromosomal *E. coli* attaching and effacing ( 

\(\text{efa1/lifA}\) \) gene that encodes for a large 385 kDa adhesion protein, called intimin. Intimin mediates attachment to epithelial cells and leads to the attaching and effacing phenotype. EPEC can be differentiated from EHEC by the absence of the shiga toxin ( 

\(\text{stx}\) \) genes that are found in EHEC. EPEC is further classified into typical and atypical strains based on the presence or absence of the *E. coli* adherence factor containing the bundle-forming pilus ( 

\(\text{bfp}\) \) gene. EPEC containing the 

\(\text{bfp}\) \) gene are classified as typical EPEC (t-EPEC) [1]. Strains lacking 

\(\text{bfp}\) \) are classified as atypical EPEC (a-EPEC).

Typical enteropathogenic *E. coli* is accepted as a diarrhea pathogen, but the pathogenicity of a-EPEC is controversial. Some studies have shown an association with a-EPEC and diarrhea while others have not. In addition, a-EPEC can be found in asymptomatic children [4–6]. Diarrhea was seen in volunteers who ingested a-EPEC but to a lesser degree than in those who ingested t-EPEC [7, 8]. This controversy surrounding a-EPEC virulence has led to a search for additional gene markers in a-EPEC that might indicate pathogenicity. The 

\(\text{efa1/lifA}\) \) gene appears to be the leading virulence candidate. 

\(\text{Efa1/lifA}\) \) encodes for a large 385 kDa adhesion protein, called the EHEC factor for adherence (*Efa1*) since it was first described in an EHEC 0157:H7 strain [9]. 

\(\text{Efa1}\) \) was found to be identical to the lymphocyte inhibitory factor A protein (*lifA*) gene that lymphostatin, which inhibits lymphocyte proliferation and lymphokine production. The designation 

\(\text{efa1/lifA}\) \) was therefore given to the gene.

In study of 182 possible virulence markers in a-EPEC cultured strains from Norwegian children, the 

\(\text{efa1/lifA}\) \) gene had the strongest statistical association with diarrhea [9]. 

\(\text{Efa1/lifA}\) \) was present in 30% of a-EPEC strains from children with diarrhea and no strains from children without diarrhea ( \(p = 0.0008\) ). In a later study from Japan, 

\(\text{efa1/lifA}\) \) was detected in 33% of a-EPEC strains in individuals with diarrhea and 13% of those in a healthy control group [10]. This difference was statistically significant ( \(p < 0.05\) ), and 

\(\text{efa1/lifA}\) \) was the only gene examined which was significantly associated with diarrhea.

These studies demonstrating the importance of 

\(\text{efa1/lifA}\) \) were performed using stool cultures for EPEC, but this is a labor-intensive process that is not feasible in clinical microbiology laboratories, since it requires isolating multiple *E. coli* colonies in each specimen and then testing these individually for the genes of interest. We therefore developed a direct fecal 

\(\text{efa1/lifA}\) \) real-time quantitative PCR (qPCR) method to determine what proportion of fecal specimens containing a-EPEC also contained 

\(\text{efa1/lifA}\) \). We then examined whether a-EPEC bacterial loads were higher in children when 

\(\text{efa1/lifA}\) \)-containing a-EPEC was present. Our hypothesis was that since the quantity of EPEC in fecal samples as measured by qPCR is associated with disease severity, infection with 

\(\text{efa1/lifA}\) \)-containing strains of a-EPEC might lead to higher bacterial loads [11].

Of note, work to clarify the pathogenicity of a-EPEC has become more crucial in recent years since EPEC are now detected by a commercially available molecular diarrheal panel, the Biofire FilmArray gastrointestinal panel (Biomerieux, Durham, NC) [2, 3]. This panel detects the 

\(\text{eae}\) \) gene, but not 

\(\text{bfp}\) \) or 

\(\text{efa1/lifA}\) \). Since a-EPEC is much more likely than t-EPEC to be present in developed countries, clinicians seeing children with diarrhea whose samples test positive for EPEC will face uncertainty as to whether the organism detected is the cause of the illness. Additional molecular biomarkers that are associated with a-EPEC pathogenicity rather than the presence of the 

\(\text{eae}\) \) gene alone may therefore be useful.

Methods

Study site and ethics approval

This was a prospective observational study performed in the months June–Aug 2010–2013 at the Children’s Hospital of Eastern Ontario, Ottawa, ON Canada, a 165 bed tertiary care hospital serving a catchment area of 1.5 million. It was decided to collect samples in the summer months since a seasonal predominance increasing in these months has been described for both EPEC and EHEC [1, 5].

Ethics approval was obtained for the study from the hospital Research Ethics Board (CHEOREB # 12/194X). Residual aliquots of fecal samples submitted for bacterial stool culture from patients with diarrhea that would otherwise have been discarded were tested by PCR for the target bacterial genes described below. All samples submitted to the laboratory for bacterial stool culture over the study period were included in the study.

The results of bacterial stool culture were recorded. Fecal samples were saved at −80 °C until nucleic acid extraction was performed. All patients were ≤18 years of age. According to the ethics approval received, in order
to ensure patient anonymity, we did not record patient age or gender.

**Outcome measures**

Our main outcome measure was the difference, if any, in a-EPEC bacterial load in efa1/lifA positive vs. negative samples. The secondary outcome measures were the prevalence of a-EPEC and t-EPEC in fecal samples; the prevalence of efa1/lifA in samples in which a-EPEC was detected; and a comparison of EPEC prevalence to the prevalence of bacterial pathogens detected by culture.

**Laboratory methods**

**Culture methods**

Fecal samples were collected in Cary-Blair enteric transport medium. Samples were inoculated onto Blood agar plate, MacConkey agar plate, Hektoen enteric agar plate, Sorbitol MacConkey agar plate, Campylobacter agar plate and Selenite broth. The Campylobacter plate was placed in a microaerophilic environment at 42 °C. Other media were incubated in ambient air at 35 °C. After overnight incubation, the selenite broth was subcultured onto a Hektoen plate. Plates were examined for Salmonella species (ssp.), Shigella ssp., Campylobacter ssp., Yersinia enterocolitica, E. coli 0157:H7, Aeromonas ssp. Plesiomonas shigelloides and Vibrio spp. Possible pathogens were identified using standard laboratory methods [12].

**PCR methods**

DNA was extracted from fecal specimens using automated device (iPrep, Life Technologies, Carlsbad, CA). Extracted DNA was then treated to remove fecal PCR inhibitors using a commercial method (Zymo-Spin™ IV-µHRC Spin Filter Zymo Research, Irvine, CA).

Primer and 5′ exonuclease probe sequences for the study assays (eae, stx1, stx2, bfp and efa1/lifA) are shown in Table 1. The efa1/lifA assay was designed for this study while other assays had been previously published. Efa1/lifA assay design was performed using a commercial qPCR program (Allele ID, Premier Biosoft, Palo Alto CA). The limit of detection of the assay was measured by duplicate testing of 10-fold serial dilutions of a synthetic oligonucleotide target sequence (Ultramer, IDT, Coralville, IA).

Probe and primer specificity were checked using Basic Local Alignment Search Tool (BLAST) sequence searches and by testing the assay against the following bacterial organisms: Streptococcus pneumoniae American type culture collection (ATCC) 49,619, Streptococcus salivarius ATCC 13,419 Escherichia coli ATCC 25,922, Haemophilus influenzae ATCC 49,766, H. influenzae ATCC 49,247, H. parainfluenzae ATCC 7901, Klebsiella pneumoniae ATCC 700,603, Moraxella catarrhalis ATCC 25,238, Staphylococcus aureus ATCC 29,247, Neisseria gonorrhoeae ATCC 49,226, N. lactamica ATCC 23,970, Pseudomonas aeruginosa ATCC 27,853, Entercoccus faecalis ATCC 29,212, S. dysgalactiae subsp. equisimilis, S. agalactiae (Group B Streptococcus), S. intermedius, S. constellatus, and S. anginosus.

QPCR was performed on all samples for the eae gene and for the stx-1 and stx-2 genes to exclude EHEC [12, 14, 15]. Eae-positive; stx-negative samples were then tested for the bfp gene to differentiate t-EPEC and a-EPEC and for the efa1/lifA gene. Probes were of the 5′-prime exonuclease type and were labelled with fluorescein amidite. Four probes were obtained from IDT (Coralville, IA) and contained an internal quencher as well as a 3′-prime end quencher. One probe contained a minor groove binder and no internal quencher and was obtained from Life Technologies (Carlsbad, CA).

PCR assays were prepared in 20 µL volumes in 96-well QPCR plate. Positive and negative controls (no template) were performed with each qPCR plate run. QPCR plates were covered with MicroAmp® Optical Adhesive Film (Life Technologies Carlsbad, CA) to prevent cross-contamination. QPCR was performed with a 96 well fast cycling block on a ViiA7 thermocycler (Life Technologies) using 40 cycles of 2-temperature thermocycling (95 °C × 3 s and 60 °C × 30 s). QPCR was considered positive for the eae, stx1, stx2, and efa1/lifA genes if the cycle threshold value was ≤35 cycles and for the bfp gene if the cycle threshold value was ≤30 cycles. Specimens

### Table 1 Gene targets PCR primer and probe sequences

| Target gene/symbol | Forward primer | Reverse primer | Probe | Reference |
|--------------------|----------------|----------------|-------|-----------|
| Intimin (eae)      | catgatcaggtttctgtctggata | ctcatcgccgaaatgctctgta | atctgcttcgcatgattgcctcagntccaat | [11] |
| Shiga toxin 1 (stx1) | gtcgcttaattctgctctgta | gctgtcgttacgttcggttctg | tcgcggcgcctgatctcgcggccctgcggct | [12] |
| Shiga toxin 2 (stx2) | ggcgctttgctgtctggta | gacaagcgtgctttgctgtctg | gatctgttcgcatgattgcctcagntccaat | [13] |
| Bundle-forming pilus (bfp) | gcatcctgcgggtgga | ggcagccgctgttgcggtttgctg | cgagtcggccagcttctggttc | [14] |
| EHEC factor for adherence 1/lymphocyte inhibitory factor A (efa1/lifA) | tacacccagaatttatacgctcaca | atgctgcttcgcatgattgcctcagntccaat | acgcggcctgtcggacttcg | This study |

*MGB* minor groove binder

* Modified from published
were classified as containing EPEC if they were eae-positive and stx-negative, and then as t-EPEC (bfp-positive) or a-EPEC (bfp-negative). A-EPEC samples were further classified as being efa1/lifA-positive or negative.

Results
The limit of detection for the efa1/lifA assay was approximately 6 copies/PCR reaction. In comparison, the detection limit for the eae assay was one log higher at 60 copies/reaction. There was no cross-reactivity observed for the efa1/lifA assay with the non-E. coli bacterial species tested.

Three-hundred twenty fecal diarrheal samples were tested. The PCR and culture results are shown in Table 2. Thirty-nine of 320 (12%) fecal samples tested were found to have the EPEC marker profile (eae-positive, stx-negative). Of these EPEC samples, 38/39 (97%) were a-EPEC (bfp-negative), with only one t-EPEC (bfp-positive).

The efa1/lifA gene was detected in 16/38 (42%) a-EPEC samples. The median eae concentration in these samples was 16,745 copies/µL (range 26–3152,879 copies/µL). For the 22 efa1/lifA-negative a-EPEC samples, the median eae concentration was 1183 copies/µL (range 9–338,770 copies/µL). The difference in median copies/µL between the two groups was statistically significant (p = 0.006, Wilcoxon two-sample test two-sided).

Bacterial cultures were positive in 27/320 samples (8.4%), with 28 organisms detected in total. Other bacterial pathogens were detected in 4/38 (10%) a-EPEC-positive samples. The most common single bacterial type identified by culture was Salmonella spp., found in 13/320 (4%) samples. Only 1 EHEC (an E. coli O157:H7) was detected by culture, while one or both stx genes were detected by qPCR in five samples, including the one culture-positive sample.

Discussion
Our findings show that EPEC genes were present with relatively high frequency in fecal samples submitted for bacterial culture from children with diarrhea in Ontario, Canada. EPEC genes were detected in more samples than the most prevalent pathogen detected by stool culture, Salmonella spp. (12 vs. 4%, respectively). This relatively high rate of detection of EPEC in developed country settings when molecular testing is used has also been reported by others. For example, in a study of daycare attendees in the Netherlands using a qPCR method, EPEC was detected in 19.9% of stool samples [6]. Investigators using the Biofire Film Array GI panel (biomerieux) reported detecting eae in 15% of diarrheal samples in a European multicenter study and 29.49% of samples in a US study [2, 3]. The proportions of t-EPEC and a-EPEC were not stated in either study (the bfp gene is not included in the Biofire panel), but most are likely to have been a-EPEC, given the low prevalence of t-EPEC in developed countries [1].

However, as discussed previously, it remains uncertain whether a-EPEC caused the diarrhea seen in these patients. Other pathogens may have been present in our patients that were not detected by bacterial stool cultures performed. It is also possible that a-EPEC may be able to grow better in the diarrheic environment created by other organisms, but is not contributing to disease.

Volunteer studies have been performed to examine a-EPEC pathogenicity but the results of these studies

Table 2 PCR and culture results from diarrheal fecal samples

| PCR                                      | No. detected (%) n = 320 |
|------------------------------------------|-------------------------|
| Eae positive                             | 44 (14)                 |
| EPEC: eae positive/stx1 and stx2 negative| 39 (12)                 |
| Atypical EPEC: eae positive/stx1 and stx2 negative/bfp negative | 38 (12) |
| Typical EPEC: eae positive/stx1 and stx2 negative/bfp positive | 1 (0.3) |
| EHEC: eae positive/stx1 or stx2 positive | 5 (1.5)                 |
| eae positive/stx negative/efa1/lifA positive/bfp negative | 16 (5) |
| eae positive/stx negative/efa1/lifA negative/bfp negative | 22 (7) |
| Culture                                  |                         |
| Salmonella spp.                          | 13 (4)                  |
| Campylobacter spp.                       | 5 (1.5)                 |
| Shigella spp.                            | 3 (0.9)                 |
| Plesiomonas shigelloides                 | 3 (0.9)                 |
| Yersinia enterocolitica                  | 2 (0.6)                 |
| E. coli O157:H7                          | 1 (0.3)                 |
| Aeromonas spp.                           | 1 (0.3)                 |

Eae, E. coli attaching and effacing gene; EPEC, enteropathogenic E. coli; stx1, shigatoxin 1 gene; stx2, shigatoxin 2 gene; bfp, bundle-forming pilus gene; EHEC, enterohemorrhagic E. coli; efa1/lifA, EHEC factor for adherence 1/lymphocyte inhibitory factor A gene
were not conclusive. For example, diarrhea occurred in 9/10 volunteers who ingested a t-EPEC strain, but in 6/11 who ingested an a-EPEC strain [7]. In a second study, 11/13 adults developed diarrhea with t-EPEC ingestion, as compared to 5/30 who ingested bfp-negative mutant strains. Thus, strains lacking bfp show reduced virulence in adults [8]. However, interpreting these studies in the pediatric setting is difficult since these adult volunteers may have been exposed to EPEC strains earlier in life and developed some degree of immunity prior to the challenge study.

More recently, a strong statistical association between the presence of efa1/lifA-positive a-EPEC and the presence of diarrhea was noted in two studies, suggesting efa1/lifA-positive strains may be true diarrhea pathogens [9, 10]. Some research has been done that provides a biological basis for the contribution of efa1/lifA to a-EPEC pathogenicity. Efa1/lifA is known to be an adherence factor and, in a study by Badea, an efa1/lifA mutant EPEC was found to be significantly less adherent to epithelial cells than the parent efa1/lifA-positive strain. Additionally, human and rabbit hosts infected with an attaching and effacing (A/E) pathogens were found to produce antibodies to efa1 protein, and anti-efa1 antibodies reduced the adherence of efa1-positive EPEC to epithelial cells [16]. These findings suggest that the efa1/lifA gene, in concert with the eae gene, may play a role in adherence of a-EPEC infections that could then lead to diarrhea.

We also observed that samples with efa1/lifA contained higher loads of a-EPEC (as measured by eae gene quantitation) than efa1/lifA-negative samples. Higher bacterial loads have been associated with occurrence of diarrhea in patients with a-EPEC, so our finding of this association between bacterial load and the presence of efa1/lifA suggests strains with efa1/lifA may be more pathogenic.

The source of a-EPEC detected in the gastrointestinal tract of children in our center is unknown. Interestingly, we detected over five times more samples with EPEC markers than EHEC markers (27 vs. 5), suggesting that children are exposed to a-EPEC than EHEC much more frequently in our region. A recent study reported that a-EPEC strains that have been associated with diarrhea were found frequently in chicken and chicken products, so it is possible that these foods may be one source of exposure [17].

There are several limitations to this pilot study that should be mentioned. First, samples from children without diarrhea were not tested, so the prevalence of a-EPEC or efa1/lifA in asymptomatic children in our region is unknown. As well, we studied samples collected over summer months, rather than year-round, so it is possible that the frequency of EPEC detection may have been different with sampling over the entire year. Our study also assumes that the genes detected by PCR belong to the same stool bacterium, rather than different bacterial strains. For example, stool samples positive for eae and efa1/lifA were assumed to contain an EPEC strain that possesses both genes. Another limitation is that PCR cannot differentiate live from dead bacteria. Thus, the detection EPEC genes could be due to dead bacteria that may have been ingested but perhaps killed during cooking. However, since methods to readily detect EPEC in culture in clinical laboratories are not available, molecular methods like PCR will likely be used as the method of choice for EPEC detection in clinical specimens, despite this drawback. Finally, we did not test fecal samples for viral or parasitic pathogens or for Clostridium difficile. Some children with EPEC detected may have had one of these micro-organisms in their fecal samples, which could suggest that EPEC may not have been the cause of the diarrhea in these cases. In future work, we plan to perform testing for these additional organisms as well as EPEC.

Our objective in the study was to assess whether efa1/lifA could be directly detected by qPCR in stool samples, and whether efa1/lifA status was related to bacterial load. We now hope to be able to perform a qPCR-based case–control study for a-EPEC and efa1/lifA that will deal with the limitations noted above.

Conclusion
Given our findings and previous reports regarding the significance of efa1/lifA, continued research into the role this gene plays in a-EPEC infection is needed. New volunteer studies comparing diarrheal symptoms in those ingesting efa1/lifA-positive and efa1/lifA-negative a-EPEC would be helpful. As noted, we hope to perform a case–control study in which eae and efa1/lifA will be examined and quantified by direct fecal qPCR in children with diarrhea and healthy controls.

As pointed out in a recent review, there are many other unanswered questions regarding EPEC [18], including the pathogenicity of a-EPEC and the optimum antibiotic treatment, if any, for patients with diarrhea in whom EPEC is detected. Historically, t-EPEC diarrhea in infants has been treated with a variety of oral antibiotics, such as gentamicin and colistin, with reported success [19] but randomized controlled trials of antibiotic treatment have not been performed. For a-EPEC, no information is available regarding antibiotic treatment. Given the ongoing shift in clinical microbiology laboratories from culture methods that do not detect EPEC to molecular detection panels that do, there is a pressing need to address these questions to help guide patient care.
Abbreviations
EPEC: enteropathogenic Escherichia coli; t-EPEC: typical EPEC; a-EPEC: atypical EPEC; EHEC: enterohemorrhagic E. coli; eae: E. coli attaching and effacing; stx: shigatoxin; bfp: bundle-forming pilus; efa1/efa2: EHEC factor for adherence/lymphocyte inhibitory factor A; qPCR: real-time quantitative PCR; species: spp.

Authors’ contributions
RS drafted the primary manuscript. NB performed statistical analyses. IM, FC, and RS contributed to study design. KL, MS, and IM participated in the performance of the laboratory testing and data analysis. All authors contributed to the preparation and revision of the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The dataset supporting the conclusions of this article is available in the Labarchives.com repository (DOI: 10.6070/H49021TS, https://mynotebook.labarchives.com/share/Slinger1/MgyuNnw4NTg5OC8yMi9UcmVlTm9kZS8yMjc2MDUyNzcxfDcyLjY). The dataset is also available as supplementary material for this article (S1 Table) (H49021TS).

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