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Article

Analysis of the Virulence and Inflammatory Markers Elicited by Enteroaggregative *Escherichia Coli* Isolated from Clinical and Non-Clinical Sources in An Experimental Infection Model, India

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Abstract

Enteroaggregative *Escherichia coli* (EAEC) are highly heterogeneous in virulence; we wanted to understand the pathogenic potential of EAEC isolated various clinical and non-clinical sources in an animal model. We infected male BALB/c mice in six mice/group with 50 EAEC isolates, isolated from clinical and non-clinical sources. We studied colonization, weight loss, stool shedding, inflammatory markers and its relationship with 21 virulence genes and phylogroups, EAEC organ burden and histopathological changes. We detected significantly more inflammatory changes and fecal lactoferrin and calprotectin levels in mice infected with EAEC isolated from symptomatic cases. In clinical EAEC isolates, the presence of chromosomal genes {(*aap* (46%), *aai*C (23.3%), and SPATEs (*pet* (13.3%), *sat* (20%), *sig*A and *pic* (6.6%)}, adhesive variants {(*agg*4A (53.3%), *agg*A (53.3%), *aaf*A (36.6%), *agg*3A (40%)} of EAEC, and master regulator gene *agg*R (66.6%) were associated with higher levels of lactoferrin and calprotectin. Also, 70% (9/13) of EAEC isolated from acute diarrheal cases bearing *chu*A (70%) in our study were assigned to group B2 (4 isolates) and D (5 isolates). Real-time PCR analysis revealed that colonization by EAEC strains from different clinical and non-clinical sources occurs up to 10-15 days of life. Even from non-diarrhoeal stools and non-clinical sources, had the potential to cause prolonged colonization, weight loss, and inflammation in the intestine though the degree varied. Moreover, better understanding of EAEC pathogenic pathways is desperately needed in different clinical scenarios.

Keywords: Enteroaggregative *Escherichia coli;* virulence; inflammatory markers; Phylogeny; Multiplex PCR

1. Introduction

Diarrhoeal illnesses are a major cause of childhood mortality worldwide. Among different pathotypes of diarrheagenic *Escherichia coli* (DEC), Enteroaggregative *E. coli* (EAEC) is gaining importance as it causes persistent and acute diarrhea both in developing and developed countries[1–3].EAEC is increasingly recognized as a common cause of diarrhea in healthy, malnourished, and immune-deficient adults and children[4–7]. The pathogenesis of EAEC is not completely understood. Several *in vitro* and *in vivo* models have been used to study the pathogenesis of EAEC[8,9]. EAEC strains express several virulence factors encoded on the chromosome or on the EAEC-specific pAA plasmid[1,10,11]. Most EAEC strains harbor a transcriptional activator called AggR that activates several factors encoded on the pAA plasmid and chromosome[12]. Based on the presence or absence of the transcriptional regulator AggR, EAEC strains are classified into typical and atypical groups, respectively. The genes under AggR control include those that encode the Aggregative Adherence Fimbriae (AAF), of which at least five variants exist[13,14]. AAFs are necessary for adherence to human intestinal explants and elicit both cytokine release and opening of epithelial cell tight junctions[15]. Among toxins, Serine Protease Autotransporters of *Enterobacteriaceae* (SPATEs) comprises a diverse group of trypsin-like serine proteases, which are produced by all of the DEC pathotypes and *Shigella* strains. SPATEs have been categorized phylogenetically into two classes. Class 1 SPATEs are cytotoxic to epithelial cells, including toxins such as plasmid-encoded toxin (Pet) and its 2 homologs, *Shigella* IgA-like protease homolog

(SigA), and secreted autotransporter toxin (Sat)[16,17].Class 2, or non-cytotoxic SPATEs, contain Pic, a mucinase that facilitates EAEC intestinal colonization, and SepA, which is a cryptic membrane protein originally identified in *Shigella* and contributes to intestinal inflammation[18]. SepA is widespread among EAEC strains[19].

EAEC may also cause inflammatory diarrhea [20]and have been shown to produce inflammatory markers like fecal lactoferrin, interleukin (IL)-8, calprotectin, and IL-1β, which are markers of intestinal inflammation[21,22].Lactoferrin is an 80 kDa iron-binding glycoprotein and is a major constituent in the secondary granules of neutrophilic leukocytes[21,23]. Its presence in body fluids, including an intestinal lumen, is proportional to the flux of neutrophils, and its assessment can provide a reliable biomarker for inflammation[24]. Calprotectin is a protein found in white blood cells, is activated when inflammation occurs, and thus can be used as a diagnostic indicator. Many factors such as demographics, phylogroups, pathogenesis, host-EAEC interactions, and clinical presentation of EAEC are associated with variation in the production of pro-inflammatory immune responses[25].It has been observed that *E. coli*harbouring the *chu*A gene and belonging to the phylogenetic groups B2 and D are mostly able to colonize the gastrointestinal tract (GIT) mucosa and to survive inside the cells[26]. A recent study has demonstrated that the presence of specific *E. coli* (both belong to groups B2 and D), in colonic biopsies, are associated with inflammatory bowel disease (IBD)[27].

Cellular and molecular mechanisms underlying initiation, progression, and outcomes of EAEC associated inflammation are largely unknown[28]. This lack of understanding could be due to strain heterogeneity and the unavailability of appropriate animal models[29]. As a result, highly reproducible animal models that enable researchers to analyze cellular responses to the intestinal mucosa during EAEC infection are still urgently needed[25].Since EAEC virulence is highly heterogeneous, and none of the virulence factors are observed in all EAEC isolates, we wanted to understand the pathophysiology of EAEC isolated from various clinical and non-clinical sources, in an animal model to determine colonization, weight loss, stool shedding, intestinal inflammatory markers, and histopathological changes induced by diverse EAEC strains. We also assessed the relationship of inflammatory markers with virulence genes and phylogroups.

2. Material and Methods

2.1. Ethics statement

The study was approved by the Postgraduate Institute of Medical Education and Research (PGIMER) Ethics Committee (INT/IEC/2017/173). All animal experiments were conducted according to the ethical guidelines approved by the institutional animal ethics committee (Ref. No.93/IAEC/650)of PGIMER.

2.2. **Animals used**

Six weeks old male BALB/c mice were provided with sterilized water and food before and during the experiments. For each isolate, 6 mice were used.

2.3. Selection of EAEC Strains

A total of fifty EAEC strains isolated from human, animal, and environmental sources were selected for animal study from our strain collection in a previously published study[3]. The pCVD432 primer was used in this study for the detection of EAEC. Among 50 EAEC strains, 30EAEC strains were isolated from humans (acute diarrhea n=13, healthy children n=7,immunocompromised patients n=4, malnourished n=3, and chronic diarrheal patients n=3). Also, ten EAEC strains from each animal and environmental sources were used for animal study. The detail of the strains and their source has been provided in supplementary data Table S1. The reference strain EAEC 042 was used as a control strain, as shown in supplementary data Table S2.

2.4. Defining EAEC strains

The EAEC strains used in this study were detected by using pCVD432 primer by using multiplex PCR which amplified the 630 bp region from start position 65 to the end position 694 of the CVD432 gene.

2.5. Infection of the mice

The infection was given to each group by following the standard protocol described by Myhal *et al*.[30]. Mice were given drinking water containing streptomycin sulfate (5g/1 sterilized water) for one day to clear their intestines of streptomycin-sensitive gut bacteria. After one day of streptomycin treatment, the number of facultative anaerobic bacteria per gram feces dropped from approximately 1X10^8 to an undetectable number on agar plates (i.e., less than 10^2 cells/g feces). After overnight starvation, each group was inoculated intragastrically with 1X10^{10} cells (0.2 ml) of the EAEC strains isolated from different sources by using a one ml tuberculin syringe fitted with a 20 gauge needle[31]. The mice were closely observed to ensure that they did not regurgitate or aspirate the given inoculum. Six mice were inoculated separately with 0.2 ml of filter-sterilized phosphate-buffered saline (PBS) and were used as control. Mice were closely monitored, and stool samples were collected daily for bacteriological analysis.

2.6. Mice Colonization Experiments:

2.6.1. **Bodyweight measurement and quantitative determination of bacterial loads**

The bodyweight before and after the oral infection was monitored on days (0, 1, 2, 4, 6, 10, 12, and 15), and the percentage change in the body weight was calculated. Briefly, the stool sample from each group was serially diluted from 10^{-1} to 10^{-7} using sterile PBS as a diluent. A 100µl of the sample was aliquoted from each tube and plated using a flame-sterilized L-shaped glass rod on MacConkey agar. Plates were incubated at 37 °C for 24 h, and the bacterial count was reported as CFU/ml.

2.7. **Analysis of inflammation markers:**

2.7.1. Detection of fecal lactoferrin and calprotectin

Stool samples of infected mice from each group were pooled after 24 hours of infection and were dissolved in one ml of PBS. Samples were centrifuged at 1000g at 2-8^0C for 20 minutes to remove insoluble impurity and cell debris at 1000g at 2-8°C. The clear supernatant was collected in the fresh vial, and the presence of fecal lactoferrin was detected using an immune chromatographic detection kit (Mouse LTF/LF (Lactoferrin) based on sandwich enzyme-linked immune-sorbent assay technology. Anti-LTF/LF antibody pre-coated 96-well plates, and the biotin conjugated anti- Anti-LTF/LF antibody was used as detection antibodies. Similarly, calprotectin in mice stool samples was detected by using an immune chromatographic detection kit (S100 Calcium Binding Protein A9) in the pre-coated Anti-S100A9 antibody 96-well plates, and the biotin conjugated anti-S100A9 antibody was used as detection antibodies.

2.8. Analysis of Stool for Bacterial Burden by real-time PCR:

The stool samples of each group of BALB/c mice infected with EAEC strains from different sources were collected on the 1st, 5th, 10th, and 15th days and cultured on MacConkey agar, and colonies of *E. coli* were identified by Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) (BrukerDaltonik GmbH, Bremen, Germany). Identified *E. coli* was further subcultured on sterile MacConkey agar and subjected to DNA extraction. The DNA extraction was performed using an optimized heat shock method. Briefly, *E. coli* strains were grown on MacConkey agar at 37^{0}C overnight. A loopful colony of bacteria was added into 100µl TE buffer and incubated at 100^{0}C for 10 min. After incubation, samples were centrifuged, and 70µl of supernatant was stored at -20^{0}C as template DNA[32].Detection and quantification of the EAEC were done using a Real-Time PCR Detection System by using the *att*A gene. The forward and reverse oligonucleotide primers for the *att*A gene as used for real-time PCR were as follows: forward, 5′-AGG TTTGATA TTGA TGTC CT TGAGGA-3′; reverse, 5′-TCA GCT AATAA TGTATAGA AAT CCG CTGTT-3.The reaction was performed by using the real-time time PCR system (Step-One^{TM} Applied biosystem). Each 40µl reaction mixture contained 25µl SYBR Green master mix (Power SYBR Green PCR master mix Applied biosystem), 2µl of template DNA, and 0.5 mM of forward and reverse primer of the target and housekeeping gene (16S rRNA). The forward and reverse oligonucleotide primers for the 16S rRNA gene used for real-time PCR were as follows: forward, 5’-CATT GACGT TACCC GCAG AAT-3’; reverse 5′-CGCT TTACGCCC AGTAAT TCC-3’. The reaction mixture was subjected to 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for the 60s. After 45 cycles, a melting curve with a ramp speed of 2.0°C/s between 70°C and 95°C was determined with a reading every 0.2°C using SYBR green fluorescence. Melting curves were analyzed using the applied biosystem software (version 2.0). Melting analysis showed a distinct peak for the target gene with melting temperatures.

2.9. Detection of virulence factors by PCR

E. coli strains confirmed as EAEC were further investigated for virulence genes. PCR for virulence genes, including *aap*, *pet*, *sig*A, *pic*, *sep*A, *sat*, *aai*C, *agg*4A, *aaf*A, *ast*A, *sep*A, *sat*, ORF3, *agg*A, *agg*3A, *aaf*C, ORF61, *eil*A, *cap*U, *air*, *esp*Y2, and *rmo*A. The list of primers used, product size, and their primer sequences are provided in Table S3 (Supplementary data).

2.10. Phylogenetic analysis via PCR

A triplex PCR was used to detect phylogenetic groups A, B1, B2, and D by amplifying the following gene targets. *chu*A, *yja*A, and a cryptic DNA fragment, TspE4C2 [33]. The classification was correlated with Clermont dichotomous decision tree [34] The list of primers used, product size, and their primer sequences are provided in Table S4 (Supplementary data).

1.11. Histopathological analysis:

To understand the pathogenesis of EAEC, one mouse from each group was sacrificed every 1st, 5th, and 10th day after infection. The rest of the mice were sacrificed on the 15th day. The intestinal tissue of each group of mice was preserved in 10% buffered formalin and dehydrated in gradient ethanol (30-100%) for histopathological analysis. Paraffin wax blocks were prepared, and thin sections were stained by hematoxylin and eosin. The pathological observation of all tissues was done through microscopy analysis[35].

1.12. Statistical analysis

A two-tailed chi-square test was used to compare groups. If low predicted values constrained the study, Fisher's exact test was used. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated using the GraphPad PRISM software.

2. Results

3.1. Mice colonization experiment:

3.1.1. Measurement of body weight changes and bacterial shedding through the Mouse Gastrointestinal Tract in Balb/c mice:

Streptomycin-treated BALB/c mice were intragastrically gavaged with EAEC strains. The bodyweight of each mice was measured on days (0, 1, 2, 4, 6, 10, 12, and 15), and the percentage change in the body weight was calculated. Colonization of EAEC strains in BALB/c mice resulted in weight loss which continued upto 5-6 days. Stool samples were collected over 1-10 days, and CFU was calculated. It revealed prolonged colonization, maximum fecal shedding of the organism was noted on day one after infection which decreased after the 5th day of infection.

3.2. Virulence-related markers and phylogeny among EAEC isolate from clinical and non-clinical sources used in the animal model.

Among clinical EAEC strains, the prevalence of chromosomal genes [*aap* (46%), *aai*C (23.3%), and SPATEs toxins), adhesive variants [*agg*4A (53.3%), *agg*A (53.3%), *agg*3A (40%), *aaf*A (36.6%) and *aff*C(33.3%)] and master regulator gene *agg*R (66.6%) was higher than EAEC isolates from non-clinical sources as shown in Table 1. In phylogeny, 70% (9/13) of EAEC strains isolated from the human source belonged to group B2 (10 isolates) and D (11 isolates), whereas EAEC strains from animal sources were belongs to phylogroup B1 (30%) and D (30%) (Table 1). Statistically, a significant difference was observed for the following virulence genes astA (p = 0.01), *aai*C (0.03), *agg*A (0.008), *aaf*A (0.001), *agg*3A (0.001), *aaf*C (0.003), *esp*Y (0.03), and *rmo* (0.005) among clinical and non-clinical EAEC isolates. However, there was no significant association among clinical and non-clinical EAEC isolates (Table 1).

Table 1. Distribution of virulence-related markers and phylogeny among EAEC isolates from clinical and non-clinical sources used in the animal model.

	Clinical isolates	**Non-clinical isolates**		
EAEC virulence gene	**Human EAEC n=30 (%)**	**Animal EAEC n=10 (%)**	**Environmental and Chutney EAEC n=10 (%)**	***P* value**
astA	29 (96.6)	5 (50)	9 (90)	0.01*
sigA	2 (6.6)	2 (20)	0	0.3
Pic	2 (6.6)	0	1 (10)	1.0
SepA	4 (13.3)	0	0	0.14
Sat	6 (20)	0	0	0.06
pet	4 (13.3)	1 (10)	0	0.6
aap	14 (46)	2 (20)	5 (50)	0.5
aaiC	7 (23.3)	0	0	0.03*
agg4A	16 (53.3)	1 (10)	5 (50)	0.14
aggA	16 (53.3)	0	3 (30)	0.008*
aafA	11 (36.6)	0	0	0.001*
agg3A	12 (40)	0	0	0.001*
affC	10 (33.3)	0	0	0.003*
ORF61	9 (30)	3 (30)	3 (30)	1.0
eilA	10	3 (30)	0	0.19
capU	8 (26.6)	1 (10)	1 (10)	0.2
air	5 (16.6)	0	0	0.07
*esp*Y	10 (33.3)	1 (10)	0	0.03*
rmo	15 (50)	1 (10)	1 (10)	0.005*
shiA	4 (13.3)	1 (10)	1 (10)	1.0
aggR	20 (66.6)	5 (50)	5 (50)	0.2
Phylogroups				
A	3 (10)	2 (20)	0	1.0
B1	6 (20)	3 (30)	2 (20)	0.7
B2	10 (33.3)	2 (20)	2 (20)	0.5
D	11 (36.6)	3 (30)	6 (60)	0.5

*Statistically significant ($p<0.05$) when clinical and non-clinical isolates were compared. Fischer’s exact test was used.

3.3. Analysis of inflammatory markers:

3.3.1. Standard curve:

The presence of lactoferrin in stool samples of infected mice was detected by using a lactoferrin ELISA kit, and the standard curve was plotted from the absorbance taken at 450nm, as shown in Figure S1 (Supplementary data).

3.3.2. Lactoferrin levels

The inflammatory analysis revealed that all the EAEC strains isolated from different sources of human origin sources release lactoferrin at different concentrations after 24 hours of EAEC infection. Lactoferrin is bactericidal to enteric pathogens, modulates the intestinal immune response, and is released by neutrophils into the stool in response to infection.

The concentration of lactoferrin was detected in all groups infected with EAEC isolates of human origin except for one strain from a healthy child (AW-412). Similarly, the concentration of lactoferrin was detected in all groups infected with EAEC isolates of animal and environmental origin except for one strain from an environmental source (Chutney) (CH-72), as shown in Figure 1 and 2.

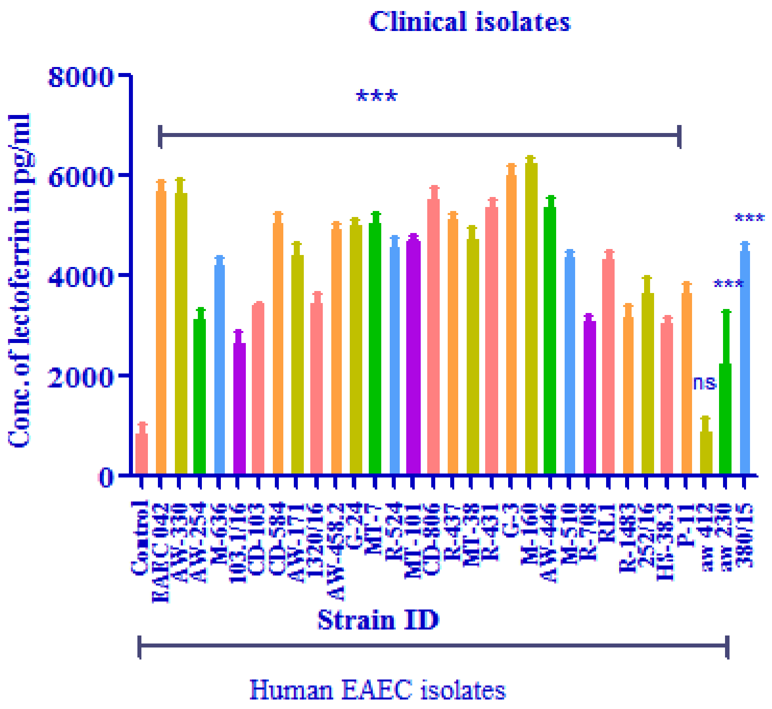

Figure 1. The concentration of lactoferrin (pg/ml) in Balb/C mice infected with the EAEC strain of human origin. *p, 0.05; **p, 0.01; ***p, 0.001; ; comparison by One way ANOVA with Dunnett's multiple comparison tests. NS= non-significant.

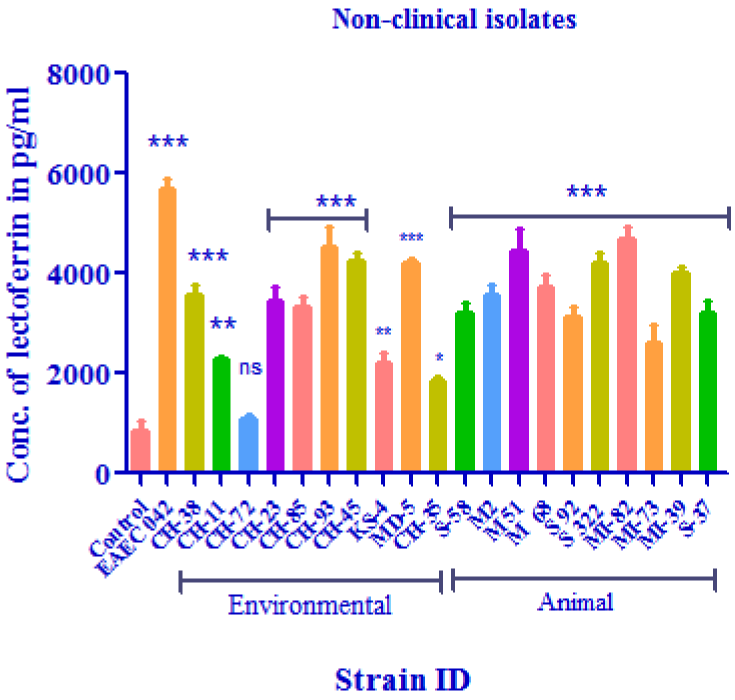

Figure 2. The concentration of lactoferrin (pg/ml) in Balb/C mice infected with the EAEC strain of environmental and animal origin. *p, 0.05; **p, 0.01; ***p, 0.001; comparison by One way ANOVA with Dunnett's multiple comparison tests. NS=non-significant.

3.4. Calprotectin detection:

3.4.1. Standard curve: Results of a typical standard run of calprotectin ELISA kit is shown below. The X-axis shows the concentration Of calprotectin, and the y-axis shows the O.D value (Figure S2 of supplementary data).

The concentration of calprotectin was detected in all groups infected with EAEC isolates of human origin. Similarly, the concentration of calprotectin was detected in all groups infected with EAEC isolates of animal and environmental origin except for two strains from environmental sources and two strains from animal sources, as shown in Figure 3 and 4.

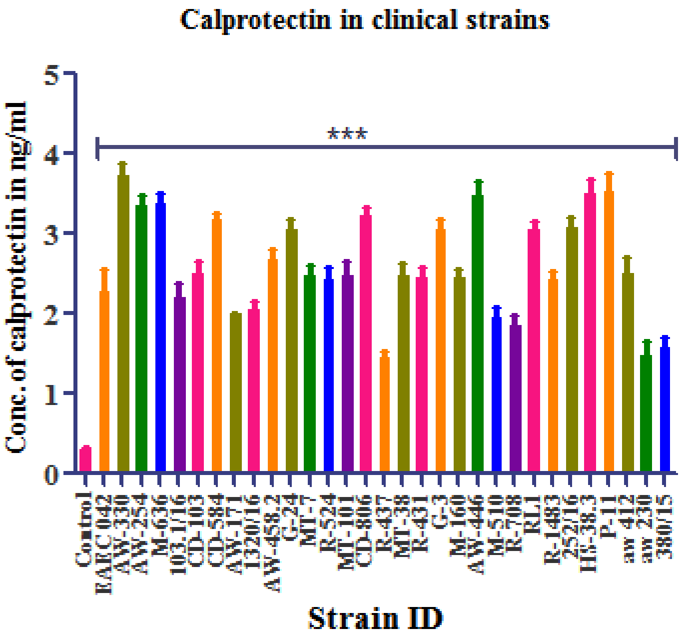
Calprotectin in clinical strains

Figure 3. The concentration of calprotectin (ng/ml) in Balb/C mice infected with the EAEC strain of human origin. *p, 0.05; **p, 0.01; ***p, 0.001; ; comparison by One way ANOVA with Dunnett's multiple comparison tests. NS=non-significant.

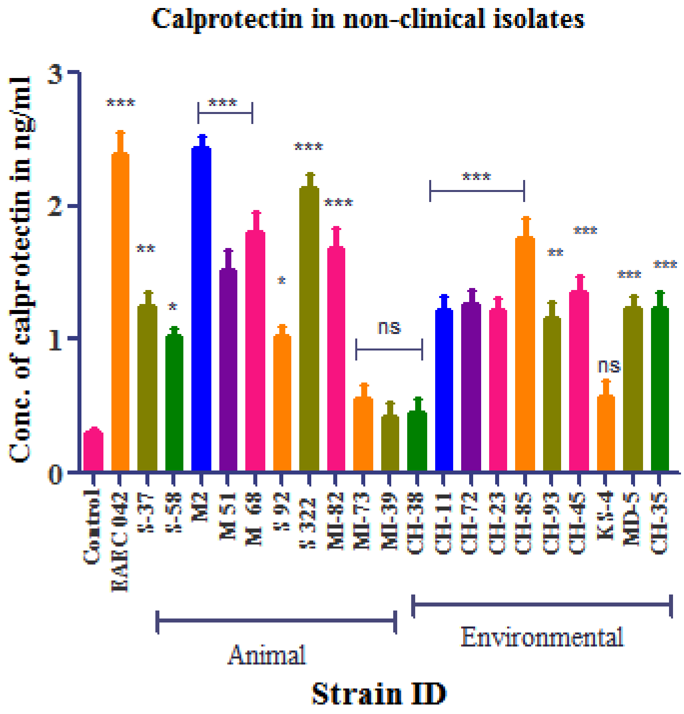
Calprotectin in non-clinical isolates

Figure 4. The concentration of calprotectin (ng/ml) in Balb/C mice infected with the EAEC strain of environmental and animal origin. *p, 0.05; **p, 0.01; ***p, 0.001; ; comparison by One way ANOVA with Dunnett's multiple comparison tests. NS=non significant.

3.5. Association of EAEC with intestinal tissue:

Despite its occurrence in stool, the quantification and time course of EAEC associated with intestinal mucosa *in vivo* is unclear. Therefore, stool samples from mice infected with EAEC strains from different sources on days 1,5,10, and 15 days were examined for the presence of the *att*A by RT-PCR. In human EAEC challenged mice, the *att*A gene was detected in stool up to 15 days of life, as compared to EAEC challenged mice from animal and environmental sources, as shown in Figure S3 (Supplementary data). Taken together these results suggest that colonization by EAEC strains from different sources (human, animal, and environmental) occurs up to 10-15 days of life.

3.6. Histopathological analysis:

In order to understand the pathogenesis of EAEC in the BALB/C mice, intestinal histopathological changes of the ileum region were analyzed on the 1st and 15th days of infection with the EAEC strain. Histopathological analysis revealed that ileum showed disrupted surface epithelium and exudates, grossly widened villous lamina propria with inflammatory cellular infiltrates [(Fig. S7 of supplementary data (B)]. Histomorphology of ileal tissue on the 15th day showed a normal appearance almost similar to that of control, as shown in Figure 5 (data C and A).

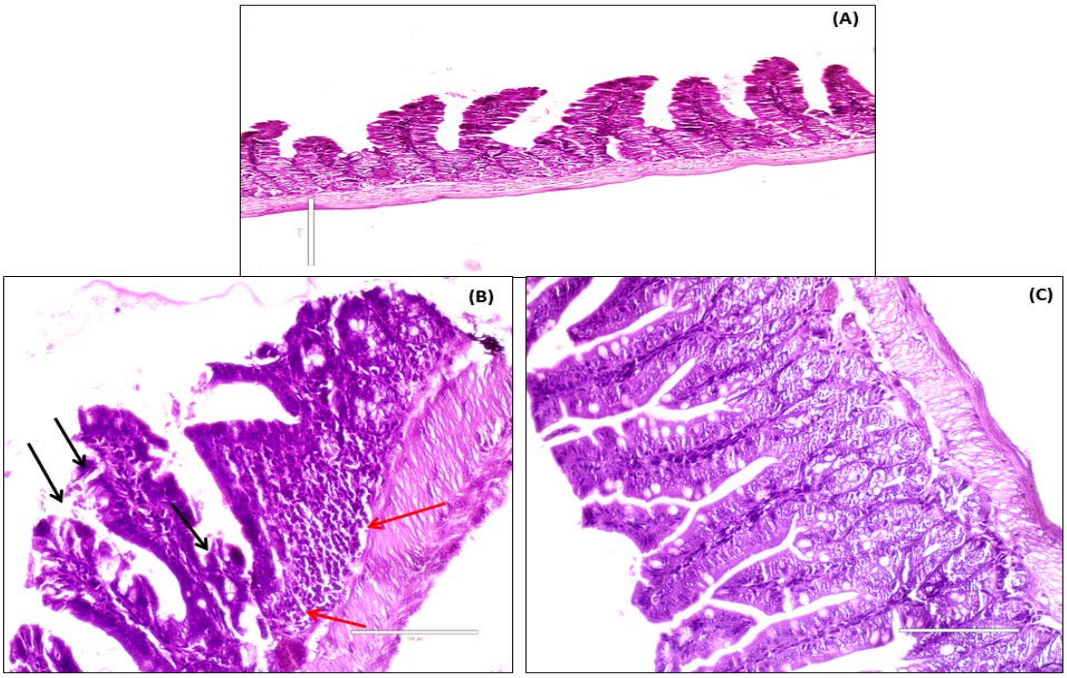

Figure 5. Shows mice intestine treated with PBS on as control (A) EAEC strain infected mice intestinal mucosa on 1st day showing disrupted surface epithelium (black arrow) and inflammatory cells (red arrow) (B) Showing mice intestine almost coming back to normal on 15th day after the challenge of EAEC strain (C

3. Discussion

EAECs are usually transmitted feco-orally via contaminated food or water[36]. Clinical manifestations of EAEC gastroenteritis include watery, mucoid, low-grade secretory diarrhea, occasionally grossly bloody stools, and little to no vomiting as evidenced in sporadic cases, outbreaks, and volunteer studies[1,37,38].Not all EAEC are pathogenic. Various animal models of EAEC infection have been used to elucidate EAEC pathogenesis[34]. Studies in isolated ligated intestinal loops of NZB rabbits, Fischer 344 rats, and 24-hour-old gnotobiotic piglets[8,39]observed multiple layers of aggregated bacteria coating an intact surface epithelium. An animal study using BALB/c mice in Kolkata, India, revealed that EAEC causes typical inflammation in the gut microvilli. The study also shows that mice could be a convenient and effective model for further study of EAEC pathogenesis. However, the study was limited to less number of mice and EAEC strains. Also, longitudinal studies of growth velocity of the host, intensity, and duration of stool shedding of the bacterium, and the total duration of bacterial association with tissue were not analyzed for EAEC causing various clinical manifestations from non-clinical sources. In our study, we have used the same model to understand and compare the virulence of EAEC isolates from human, animal, and diverse sources. We have assessed and compared the colonization and inflammatory markers produced by EAEC.

Various reports from Brazilian children infected with EAEC[20,21,40] haveshown the presence of fecal lactoferrin and pro-inflammatory cytokines, IL-1βand interleukin (IL)-8. Nataro *et al.*also reported that infection with EAEC in patients leads to higher IL-1β to IL-1ra proportions than control subjects[41]. Elevated fecal levels of IL-8, IL-1β, and IL-1ra have been observed with EAEC-associated diarrhea in travelers from India[42]. In the present study, when we looked at the related fecal lactoferrin and calprotectin levels, symptomatic EAEC infection was associated with the most severe inflammatory response in mice. Moreover, EAEC strains from non-diarrheal children without diarrhea also had elevated fecal lactoferrin and calprotectin concentrations except for one strain. Our results are supported by a study from Southern Ghana, where a high lactoferrin level was detected in diarrheal and non-diarrheal children who had EAEC infection[43]. However, the mean levels of lactoferrin and calprotectin in non-diarrheal EAEC isolated strains were lower than in diarrheal EAEC in our study. Our results were contradictory to the analysis of inflammatory markers in travelers returning from India, where no inflammatory markers were detected in commensal EAEC from stools[44]. It has been postulated that the presence of EAEC in the stool significantly impairs the growth, regardless of the presence or absence of diarrhea[45]. This is proved experimentally by Roche *et al.* in the neonatal and weaned mice model where EAEC infection causes growth shortfalls and triggers severe undernutrition[4].In our study, similar colonization and weight loss were produced by EAEC isolated from non-diarrheal stools.

In our findings, non-clinical EAEC isolates had a lower concentration of fecal lactoferrin and calprotectin than EAEC from clinical sources. One possible cause for the difference in gastrointestinal markers of EAEC-associated diarrhea infection is that not all strains are infective[21]. An earlier study has shown that virulent species elicit a particular inflammatory response throughout infection, while less pathogenic strains do not elicit a particular inflammatory response during disease[40]. Many plasmid-coded virulence factors that clarify the pathogenic ability of EAEC strains have recently been identified[46]. These include adhesive variants (AAF/I-IV), which are known to play a role in the adhesion of the mucosa; *pet* (plasmid-coded toxin); *aai*C (secreted protein); EAST-1 (heat-stable enterotoxin); and *agg*R (master regulator gene) are found to cause the inflammation[47]. We found that between diarrhea and non-diarrhea stool isolates, the presence of chromosomal genes (*aap*, *aai*C, and SPATEs), adhesive variants of EAEC, and master regulator gene *agg*R was associated with higher levels of lactoferrin and calprotectin. Additionally, more virulence EAEC genes detected in diarrheal EAEC isolates corresponded to a rise in the fecal lactoferrin and calprotectin levels. In the non-clinical EAEC isolates, the presence of a lower number of virulence genes could be responsible for lower virulence in our mice model. In Nigerian children with EAEC infection, differing levels of virulence were also seen where more EAEC virulence genes corresponded to an increase in the fecal lactoferrin level[48]. Virulence factors are associated with elevated levels of fecal cytokines and inflammatory markers such as interleukin (IL)-1ra, IL-1β, IL-8, interferon (INF)-γ, lactoferrin, fecal leukocytes, and occult blood[22]. EAEC carrying virulence factors are not always associated with the disorder. Factors such as host genetic susceptibility, host immune response, heterogeneity of virulence among EAEC strains, and the number of bacteria consumed by the infected host are responsible for the myriad variation of EAEC inflammation.

In IBD patients, a correlation between *E. coli* containing the *chu*A gene has been identified[47]. These strains were mostly able to colonize the GIT mucosa and survive inside the epithelial cells[26]. Seventy percent (9/13) of EAEC isolated from acute diarrheal cases in our study were assigned to group B2 (4 isolates) and D (5 isolates). Further, a previous study confirmed that the mean calprotectin levels were significantly increased in IBD patients colonized with *E. coli* of the B2 phylogenetic group. It also demonstrated that patients colonized with *E. coli* belonging to phylogroup B2 had significantly increased inflammation as compared to patients colonized with *E. coli* of groups D and A[49]. These results were in agreement with the results of the present study, where a high level of inflammatory markers was found with group D, followed by group B2, group B1, and then group A[50].

However, the results of our study demonstrated higher virulence scores with group B2 in clinical isolates. Smaller numbers of isolates in non-clinical isolates belonging to phylogroup B2 and carrying fewer virulence genes may be responsible for lower virulence in the animal model.

Concurrently, we find that some of the isolates that were obtained from animal and environmental sources do not cause severe disease in our mice model. EAEC infection results in functional and anatomical changes in intestinal epithelial cells following invasion and adherence to the gut of BALB/c mice. Using this mice model, the oral challenge with EAEC strains was associated with growth shortfalls and persistent shedding of organisms over 14 days. We found that the ability of EAEC strains to infect mice with EAEC, resulting in growth shortfalls and prolonged stool shedding, similar to the Baltimore study[45]. The appearance of inflammatory markers in the stool is indicative of intrusive etiology, and inflammatory diarrhea can be calculated on the basis of this assessment. Defective surface mucosa with inflammatory infiltrates in lamina propria and submucosa was revealed by histological sections of ileal tissue.On the 5th day after infection, maximum fecal shedding was noted, and histopathological changes confirmed this observation. In the mice model, fecal shedding of the organisms and histological modifications encourage moderate inflammation. After the 7th day after the infection, the normal recovery of animals occurred in due course of time.

In conclusion, this study revealed that EAEC, even from non-diarrhoeal stools and non-clinical sources, had the potential to cause prolonged colonization, weight loss, and inflammation in the intestine through the degree varied. Moreover, a better understanding of EAEC pathogenic pathways is desperately needed in different clinical scenarios. The animal model of EAEC infection used in this study would be a significant step in this effort.

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Declarations

Conflict of interest: The authors have declared that no conflict of interest exists.

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Supplementary Data:

Analysis of the virulence and inflammatory markers elicited by Enteroaggregative *Escherichia coli* isolated from clinical and non-clinical sources in an experimental infection model, India

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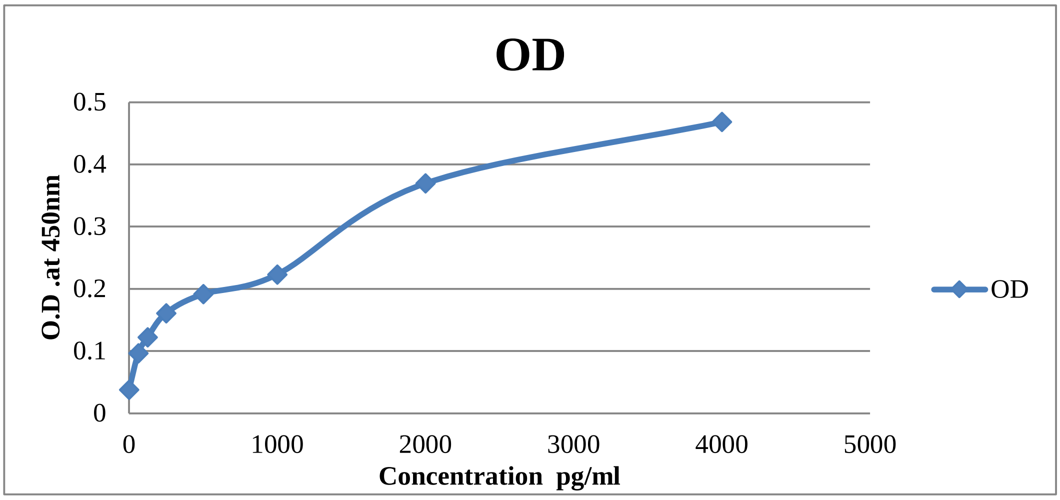

Figure S1. Standard curve: Results of a typical standard run of lactoferrin ELISA kit is shown above. The X-axis shows the concentration of the lactoferrin, and the y-axis shows the O.D value.

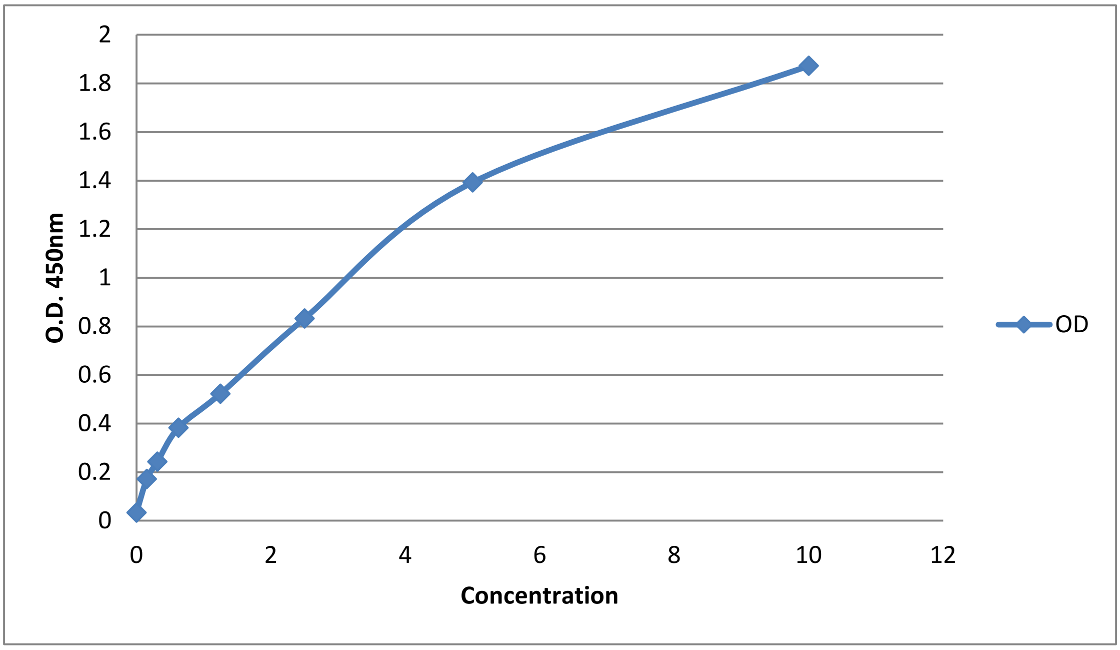

Figure S2. Standard curve: Results of a typical standard run of calprotectin ELISA kit is shown below. The X-axis shows the concentration of calprotectin, and the y-axis shows the O.D value.

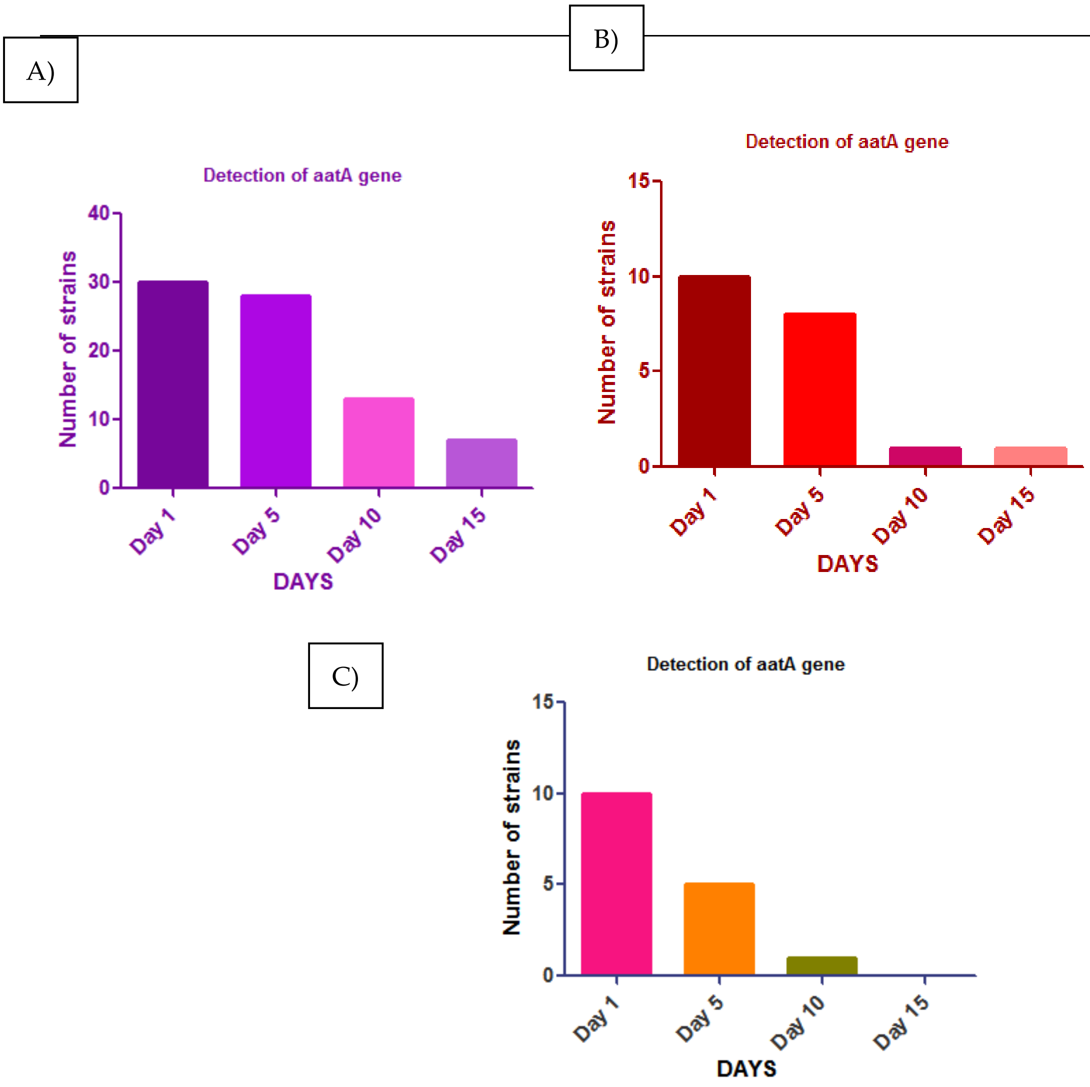

Figure S3. Colonization and persistence of EAEC (detected by *aat*A gene of EAEC) isolate in Balb/C mice from A) human origin, B) animal origin and C) environmental origin.

Table S1. The detail of the EAEC strains used in this study and their source.

Serial number	**Source**	**Strain given to mice**	**Source of strain**
1		AW-330	Healthy
2		AW-254	Healthy
3		M-636	Acute diarrhea
4		G3	Immunocompromized
5		CD-103	Chronic diarrhea
6		CD-584	Chronic diarrhea
7		AW-171	Healthy
8		1518/16	Immunocompromized
9		AW-458.2	Healthy

10	Human EAEC isolates	G-24	Immunocompromized
11		MT-7	Malnutrition
12		S 752.2	acute diarrhea
13		MT-101	Malnutrition
14		CD-806	Chronic diarrhea
15		HS-38.4	Acute diarrhea
16		MT-38	Malnutrition
17		A-22.1	Acute diarrhea
18		S-123	Acute diarrhea
19		M-160	Acute diarrhea
20		AW-446	Healthy
21		M-510	Acute diarrhea
22		526/05	Acute diarrhea
23		M-564	Acute diarrhea
24		R-1485	Acute diarrhea
25		252/16	Immunocompromized
26		HS-38.3	Acute diarrhea
27		P-30	Acute diarrhea
28		aw 412	Healthy
29		aw 230	Healthy
30		M-381	Acute diarrhea
31	Animal EAEC isolates	S-37	Animal stool
32		S-58	Animal stool
33		M2	Meat
34		M 51	Meat
35		M 68	Meat
36		S 92	Animal stool
37		S 322	Animal stool
38		MI-82	Milk
39		MI-73	Milk
40		MI-39	Milk
41		CH-38	Environmental
42		CH-11	Environmental

43	Environmental EAEC isolates	CH-72	Environmental
44		CH-23	Environmental
45		CH-85	Environmental
46		CH-93	Environmental
47		CH-45	Environmental
48		KS-4	Environmental
49		MD-5	Environmental
50		CH-35	Environmental

Table S2: Shows the group of mice to be tested.

			Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Total**
Animal	**Route**	**Years wise break-up**	**Control (PBS)**	**Control strain EAEC 042**	**EAEC strains from human source (n=30) {Acute diarhea (n=13), healthy (n=7), immunocompromised patients (n=7), malnourished children (n=3), chronic diarrhea (n=3)}**	**EAEC strains from animal source (10 strains)**	**EAEC strains from environmental source (10 strains)**	
Six week old male BALB/c	Intragastrically	1st year	6 mice	6 mice	6x30=180 mice	6x10=60 mice	6x10=60 mice	312 mice

Table S3. Primers with description of the target gene, product size in base pairs, and annealing temperature, used for the 4 multiplex polymerase chain reactions (M-PCRs) and 3 monoplex PCRs.

Target gene and description	**Type of PCR**	**Primer Sequence (5’- 3’)**	**PCR Product, Size (bp)**	**GenBank Accession No.**	**Annealing Temperature (ºC)**	**Refrences**
*ast*A- EAST-1 heat-stable toxin	M-PCR 1	ATGCCATCAACACAGTAT GCGAGTGACGGCTTTGTAGT	110	L11241	58	33
Pet- Plasmid-encoded toxin		GGCACAGAATAAAGGGGTGTTT CCTCTTGTTTCCACGACATAC	302	AF056581	58	34
*sig*A- IgA protease-like homolog		CCGACTTCTCACTTTCTCCCG CCATCCAGCTGCATAGTGTTTG	430	NC_004337	58	35
pic- Serine protease precursor		ACTGGATCTTAAGGCTCAGGAT GACTTAATGTCACTGTTCAGCG	572	AF097644	58	34
*sep*A- Shigella extracellular protease		GCAGTGGAAATATGATGCGGC TTGTTCAGATCGGAGAAGAACG	794	Z48219	58	34
sat- Secreted autotransporter toxin		TCAGAAGCTCAGCGAATCATTG CCATTATCACCAGTAAAACGCACC	932	AE014075	58	35
ORF3-Cryptic protein	M-PCR 2	CAGCAACCATCGCATTTCTA CGCATCTTTCAATACCTCCA	121	AB261016.2	57	35
aap-Dispersin, antiaggregation protein		GGACCCGTCCCAATGTATAA CCATTCGGTTAGAGCACGAT	250	Z32523	57	35

*aai*C- AaiC, secreted protein		TGGTGACTACTTTGATGGACATTGT GACACTCTCTTCTGGGGTAAACGA	313	AB255435.1	57	35
*agg*R-Transcriptional activator		GCAATCAGATTAARCAGCGATACA CATTCTTGATTGCATAAGGATCTGG	426	Z18751	57	35
*agg*4A - AAF/IV fimbrial subunit	M-PCR 3	TGAGTTGTGGGGCTAYCTGGA CACCATAAGCCGCCAAATAAGC	169	EU637023	57	35
*agg*A - AAF/I fimbrial subunit		TCTATCTRGGGGGGCTAACGCT ACCTGTTCCCCATAACCAGACC	220	Y18149	57	35
*aaf*A - AAF/II fimbrial subunit		CTACTTTATTATCAAGTGGAGCCGCTA GGAGAGGCCAGAGTGAATCCTG	289	AY344586	57	35
*agg3*A- AAF/III fimbrial subunit		CCAGTTATTACAGGGTAACAAGGGAA TTGGTCTGGAATAACAACTTGAACG	370	AF411067	57	35
*aaf*C -Usher, AAF/II assembly unit		ACAGCCTGCGGTCAAAAGC GCTTACGGGTACGAGTTTTACGG	491	AF114828	57	35
ORF61- Plasmid-encoded hemolysin	M-PCR 4	AGCTCTGGAAACTGGCCTCT AACCGTCCTGATTTCTGCTT	108	J02459.1	57	35
*eil*A- Salmonella HilA homolog		AGGTCTGGAGCGCGAGTGTT GTAAAACGGTATCCACGACC	248	CP009685.1	57	35
*cap*U- Hexosyltransferase homolog		CAGGCTGTTGCTCAAATGAA GTTCGACATCCTTCCTGCTC	395	AF134403	57	35

air- Enteroaggregative immunoglobulin repeat protein		TTATCCTGGTCTGTCTCAAT GGTTAAATCGCTGGTTTCT	600	CP009685.1	57	35
*esp*Y2- Non-LEE-encoded type III secreted effector	Singleplex PCR	CGCAAAAGATCCGGAAAATA TCAGCATTGCTCAGGTCAAC	216	ECSP_0073	57	35
*rmo*A- Putative hemolysin expression- modulating protein	Singleplex PCR	TTACCTTACATATTTCCATATC CGAAAACAAAACAGGAATGG	210	ECUMN_0072	59	35
*shi*A- shiA-like inflammation suppressor	Singleplex PCR	CAGAATGCCCCGCGTAAGGC CACTGAAGGCTCGCTCATGATCGCCG	292	ECB_03517	57	36

Table S4: Primers used for the triplex polymerase chain reactions target gene description, base-pair size, annealing temperature, and primers concentration.

Gene/ Target	**Primer Sequence (5'- 3')**	**PCR Product, bp**	**Annealing Temperature Primer Concentration (_C), pmol/µl**	**References**
ChuA	GACGAACCAACGGTCAGGT TGCCGCCAGTACCAAAGAA	279	55/25	39
YjaA	TGAAGTGTCAGGAGACGCTG GAGTAATGTCGGGGCATTCA	211	55/25	39
TspE4C2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152	52/25	39