Escherichia coli Expressing EAST1 Toxin Did Not Cause an Increase of cAMP or cGMP Levels in Cells, and No Diarrhea in 5-Day Old Gnotobiotic Pigs

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Abstract

Background: Enterotoxigenic Escherichia coli (ETEC) strains are the leading bacterial cause of diarrhea to humans and farm animals. These ETEC strains produce heat-labile toxin (LT) and/or heat-stable toxins that include type I (STa), type II (STb), and enteroaggregative heat-stable toxin 1 (EAST1). LT, STa, and STb (in pigs) are proven the virulence determinants in ETEC diarrhea. However, significance of EAST1 in ETEC-associated diarrheal has not been determined, even though EAST1 is highly prevalent among ETEC strains.

Methodology/Principal Findings: In this study, we constructed E. coli strains to express EAST1 toxin as the only toxin and studied them in cell lines and five-day old gnotobiotic piglets to determine significance of EAST1 toxin. Data from in vitro studies indicated that EAST1 did not stimulate an increase of intracellular cyclic AMP or GMP levels in T-84 cells or porcine cell line IPEC-J2, nor did it enhance LT or STa toxin of ETEC strains in stimulation of cAMP or cGMP in T-84 cells. In addition, 5-day old gnotobiotic pigs challenged with E. coli strains expressing EAST1 as the only toxin did not developed diarrhea or signs of clinical disease during 72 h post-inoculation.

Conclusion/Significance: Results from this study indicated that EAST1 alone is not sufficient to cause diarrhea in five-day old gnotobiotic pigs, and suggest that EAST1 likely is not a virulence determinant in ETEC-associated diarrhea.

Introduction

Enterotoxigenic Escherichia coli (ETEC) strains are the most common bacterial cause of diarrhea. Diarrhea is the second leading cause of death to children younger than 5 years and also one of the most important diseases in young farm animals [1–3]. The key virulence factors of ETEC in diarrhea are bacterial adhesins and enterotoxins [4]. Adhesins mediate ETEC bacteria attachment to receptors at host epithelial cells and subsequent colonization at small intestines; whereas enterotoxins disrupt fluid homeostasis in host small intestinal epithelial cells to stimulate an increase of intracellular cyclic AMP or GMP levels and to cause electrolyte-rich fluid hyper-secretion that leads to diarrhea [4]. Enterotoxins produced by ETEC strains associated with diarrhea are heat-labile toxin (LT) and heat-stable toxins (ST) that include type I (STa), type II (STb), and enteroaggregative heat-stable toxin 1 (EAST1) [4–6]. While LT, STb, and STa have been identified as virulence determinants in diarrhea [4,7–10], virulence significance of EAST1 in ETEC-associated diarrhea is unclear [11,12]. The lack of determination in significance of EAST1 in ETEC-associated diarrhea causes debate regarding whether this toxin should be targeted in disease prevention, thus diffusing efforts in developing effective vaccines against ETEC diarrhea.

Contradictory observation was reported regarding the role of EAST1 that could play in diarrhea. E. coli or enteroaggregative E. coli (EAEC) strains expressing EAST1 as the only toxin were reported to cause human diarrheal outbreaks in Japan and Chile [13–15], and a followed case-control study suggested EAST1 as a putative agent of EAEC-associated diarrheal disease [16,17]. In addition, epidemiological and clinical studies showed that E. coli strains isolated from travelers with diarrhea were commonly EAST1 gene positive [18–21]. However, the EAST1 gene was also detected in E. coli strains isolated from healthy or asymptomatic children and adults [15–17,19,20,22–25]. Similarly to the prevalence in E. coli isolated from humans, the EAST1 gene was commonly detected among ETEC strains isolated from pigs with neonatal or post-weaning diarrhea [5,6,25–31], but was also found highly prevalent in E. coli strains isolated from pigs showing no signs of diarrhea [28]. There was one study reported that some newborn gnotobiotic piglets developed diarrhea after being inoculated with EAST1-positive EAEC strains, but clinical outcomes among the challenged piglets varied substantially and
remained rather not conclusively [32], and results form that solitary pig study have never been verified [12].

Although it was initially detected among EAEC strains, EAST1 is highly prevalent among enterohemorrhagic E. coli (EHEC), ETEC and enteropathogenic E. coli (EPEC) strains [23,33-37]. A recent study reported that 35% of E. coli strains isolated from young pigs with diarrhea carried the EAST1 gene alone or together with other toxin genes [6]. However, the role EAST1 playing in diarrhea caused by EAST1-positive ETEC, EHEC or EPEC strains has not been determined. There is evidence that porcine ETEC strains trend acquiring additional virulence factors [29]. Whether EAST1 plays a synergistic role to LT and other ST toxins of ETEC strains in virulence is also unknown.

Significance of EAST1 in diarrhea, especially ETEC-associated diarrhea, needs to be determined. EAST1 antigen has to be included as an ETEC vaccine component if EAST1 is a virulence determinant in ETEC-associated diarrhea. In this study, we cloned the EAST1 gene and constructed recombinant E. coli strains to express EAST1 as the only toxin, and examined the virulence significance of EAST1 in cell lines and a pig challenge model. In addition, we examined whether EAST1 enhances LT or STa toxin of ETEC strains in stimulating intracellular cAMP and cGMP and plays a synergistic role in ETEC associated diarrhea.

Materials and Methods

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Total genomic DNA from porcine ETEC field isolate 3030-2 (K88lac/LT/STb/EAST1) was used as templates in PCR to amplify the astA gene encoding EAST1. Non-pathogenic porcine E. coli field isolate G58-1 [38], which does not carry any known toxin or fimbrial adhesion genes but can harbor plasmids to express and secrete fimbriae and toxins including LT and STa in vitro and in vivo, was used to construct E. coli recombinant strains carrying different EAST1-positive plasmids. Three vectors, the single-copy pBelo-BAC, medium-copy pBR322 and high-copy pUC19, were used to clone and express the astA gene. Enteroaggregative E. coli isolate 17-2 that naturally carries the EAST1 gene was transformed to express an additional LT or STa toxin, or K88ac fimbriae. Constructed strains were cultured in LB or 4AA medium supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), or tetracycline (12 μg/ml).

EAST1 Gene Cloning

To determine whether gene copy-number correlates to virulence, this astA gene was cloned into different expression vectors. The astA gene that was PCR amplified with primers pUC:EAST1-F (5'-atgctggtagattcattgctgg-3') and pUC:EAST1-R (5'-tcggtgatgataaatcgctg-3') was cloned into pUC19 at the HindIII and BamHI sites, with primers pBR:EAST1-F (5'-atatccttgccagttatgcatcgt-3') and pBR:EAST1-R (5'-gctgctgatgataaatcctttg-3') was cloned into pBR322 at the NheI and SpH1 sites, and with primers pBelo:EAST1-F (5'-atgctggtagattcattgctgg-3') and pBelo:EAST1-R (5'-gctgctgatgataaatcctttg-3') was cloned into pBelo-BAC11 at the HindIII and SpH1 sites, respectively. The amplified astA gene products were separated with gel electrophoresis and purified with a QIAAGEN gel extraction kit (QIAAGEN, Valencia, CA), digested with restriction enzymes, and cloned into pUC19, pBR322, and pBelo-BAC vectors with T4 ligase (New England Biolabs, Ipswich, MA), respectively. The avirulent E. coli field isolate G58-1 was transformed to express K88ac fimbriae, and the resultant E. coli (G58/K88) was used as a host strain to be further transformed with astA gene positive plasmids for isogenic strains. These isogenic strains expressed the EAST1 gene in different copy-numbers and were used for the cell line and pig challenge studies. Purification, digestion, cloning and transformation were conducted by following standard protocols [39]. Antibiotics selected transformates were DNA sequenced to verify the cloned astA gene in correct reading frame.

Quantitative PCR to Detect astA mRNA

Total mRNA from each recombinant strain was extracted using an RNAsy Mini Kit (QIAGEN). An equal amount of mRNA extracted from each strain was used in RT-PCR with the GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, CA). Five microliters of each RT-PCR product were used in quantitative PCR (in triplicate) using a TaqMan probe (5'-FAM-ccgcatcagttatgctggatgga-TAMRA-3'), PCR primers astA-F (5'-atgccacacacagctgac3') and astA-R (5'-tcaggctggaggg-3'), and gold-tag DNA polymerase (Applied Biosystems) in a Mx3000 QPCR system (Stratagene, La Jolla, CA) or a SmartCycler system (Cepheid, Sunnyvale, CA).

EAST1 Competitive ELISA to Measure Expressed EAST1 Proteins

EAST1 competitive ELISA, which was developed by Robertson laboratory, was used to examine expression of EAST1 proteins from the constructed strains. Synthetic EAST1, with an average mass of 4109 (±0.25%) and a purity over 90% (Pi proteomics, Huntsville, AL) was chemically conjugated to ovalbumin (Sigma). Ten nanogram EAST1-ovalbumin conjugates (from Grupper laboratory) were used to coat each well of a microtiter plate (Costar Cat. #2595; Corning, Corning, NY) and incubated at 37°C overnight. Coated plates were blocked with 2.5% casein buffer at 37°C for 2 h. Seventy-five microliters of culture supernatants, which were resulted from centrifugation of overnight grown culture (LB or 4AA) with equal amounts of cells from each strain, were mixed with 75 μl of anti-EAST1- chicken serum (1:1000 dilution; GenWay Biotech, Inc., San Diego, CA), and the 150 μl mixture was added to each well and further incubated at 37°C for 1 h. The plate was washed with STa ELISA washing buffer [10], and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-chicken IgY (1:2500; GenWay Biotech, Inc.). Optical density (OD) values were measured at 405 nm wavelength by a plate reader, after 20 min of reaction in a peroxidase substrate (KPL, Gaithersburg, MD).

CAMP and cGMP ELISAs to Measure Enterotoxicity of EAST1

Enterotoxicity of LT and STa of ETEC strains can be measured through stimulation of intracellular cyclic AMP or cGMP in epithelial cells. To determine whether EAST1 possesses similar biological enterotoxicity, we examined field strain 17-2 (EAST1) and EAST1-positive recombinant strains for stimulation of cAMP or cGMP levels in human cell line T-84 (ATCC, CCL-248™) and porcine epithelial cells line IPEC-J2 (a gift from Dr. Anthony Blikslager at North Carolina State University, Raleigh, NC) using an ELIA CAMP ELISA kit and an ELIA cGMP ELISA kit (Assay Designs, Ann Arbor, MI). Overnight grown cultures containing equal amounts of cells, which was calculated based on culture OD values, propagated in 4AA medium from each strain were centrifuged at 3500 rpm for 30 min. One hundred and fifty microliters of resultant supernatants were added to each well containing 1–2×105 T-84 cells or the IPEC-J2 cells. After 1 h incubation, intracellular cAMP and cGMP concentrations in the
Ussing Chamber Assay

Enterotoxicity of EAST1 expressed by the recombinant strains was also examined in Ussing chamber assay. Overnight culture growth of each strain was centrifuged, and 2 million cells each of supernatant were added to chambers containing monolayers of T-84 or IPEC-J2 cells as positive controls. Short-circuit current (Isc), which represents the algebraic sum of active ion-transport processes of all epithelial cells, was measured using a modified Ussing chamber (model DCV9, Navicyte, San Diego, CA) and MP100A-CE interface.

Gnotobiotic Piglet Challenge Study

K88ac receptor-positive piglets delivered by Cesarean-section and raised under germ-free conditions were used in the challenge study. Fecal swap samples were collected daily to monitor sterilization. At day 5, a group of 4 piglets were orally inoculated with 3×10⁹ CFUs of each inoculated strain. For brush border preparation, an ileal segment from each piglet was cut open, rinsed with hypertonic EDTA to remove feces, and incubated in hypertonic PBS, and plated on LB plates. Colonies were counted after overnight growth at 37°C. Pig small intestinal samples collected at necropsy were used to measure bacteria colonization using quantitative colonization assay [9], and to prepare brush border vesicles to confirm expression of K88ac receptors [42].

Table 1. E. coli strains and plasmids used in this study.

| Strains and plasmids | Relevant properties | Reference |
|----------------------|---------------------|-----------|
| Strains              |                     |           |
| 3030-2               | porcine ETEC isolate, K88ac/LT/STb/EAST1 | [38]     |
| GS8-1                | porcine nonpathogenic E. coli isolate | [38]     |
| 8231                 | pK88ac in GS8-1     | this study|
| 8321                 | pUC EAST1 in 8231   | this study|
| 8322                 | pBR EAST1 in 8231   | this study|
| 8323                 | pBelo EAST1 in 8231 | this study|
| 17-2                 | EAST1+ E. coli field isolate | 13       |
| 8722                 | pK88ac in 17-2      | this study|
| 8724                 | pB607 in 17-2       | this study|
| 8725                 | pB295 in 17-2       | this study|
| TOP10                | F’ mcrAΔ(ncr-hsdRMS-mcrBC) Ph80 lacZ,MM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU gshK rpsL1 [Str” endA1 nupG | Invitrogen|
| 8295                 | pB295 (STa) in TOP 10 | this study|
| 8607                 | pB607 (LT) in TOP 10 | this study|
| Plasmids             |                     |           |
| pK88ac               | K88ac fimbrial operon in pACYC177 | this study|
| pUC EAST1            | astA gene coding EAST1 in pUC19 | this study|
| pBR EAST1            | astA gene coding EAST1 in pBR322 | this study|
| pBelo EAST1          | astA gene coding EAST1 in pBelo-BAC11 | this study|
| pB295                | estA gene coding STa in pBR322 | this study|
| pB607                | eltAB genes coding porcine LT in pBR322 | this study|

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neutral buffered Formalin. Fixed tissue was embedded, sectioned, H&E stained, and examined for bacterial colonization microscopically. The animal study complied with the Animal Welfare Act by following the 1996 National Research Council guidelines [43] and was approved and supervised by a state veterinarian and South Dakota State University’s Institutional Animal Care and Use Committee.

Statistical Analysis

Data were analyzed by using the mixed procedure (SAS for windows, version 8; SAS Institute, Cary, N.C.), adjusted for multiple comparison by Bonferroni. Results were expressed as means ± standard deviations. Student’s t-test was used to compare different treatment groups. Calculated p values of <0.05 were regarded as significant when treatments were compared at two-tailed distribution and two-sample equal or unequal variance. In addition, the means procedure (H₀: r = 1; H₁: r ≠ 1) was used to analyze data from multiple independent experiments to test whether EAST1 had significant synergistic effect on LT and STa toxins in stimulation of intracellular cAMP and cGMP levels.

Results

Recombinant E. Coli Strains were Constructed

Nine E. coli strains were constructed in this study (Table 1). The astA gene, which was isolated from the porcine ETEC strain 3030-2 and had identical DNA sequence to the astA gene of EAEC prototype strain O42, was cloned in the high-copy vector pUC19, the medium-copy vector pBR322, and the single-copy vector pBelo-BAC11.

Gene amplicons from strains 8321, 8322, 8323 were correlated accordingly to the astA-gene plasmid copy numbers in these strains. Strains 8321, 8322 and 8323. The initial purpose to construct these three strains was to examine whether astA gene copy numbers or EAST1 production levels dictate virulence significance of EAST1 in diarrhea. Strain 8321 was generated from transformation of the avirulent K88 fimbrial E. coli strain 8231(-) to express EAST1 (in pUC19 vector), and strain 8722 of field isolate 17-2 to express K88ac fimbriae. These two strains were used in the piglet challenge study to examine whether an E. coli strain expressing EAST1 as the only toxin is sufficiently virulent to cause diarrhea in neonatal piglets. Strains 8724 and 8725 were derived from transformation of strain 17-2 with LT plasmid p8607 and STa plasmid p8295 to express additional LT and STa, respectively, and were used for comparative studies in EIA ELISAs to determine whether EAST1 plays a synergistic role to LT or STa toxin in stimulating intracellular cAMP and cGMP in vitro.

Different astA Gene Copy Numbers were Detected Among Recombinant EAST1 Strains

An equal amount of total mRNA from 17-2, 8722(17-2/K88ac), 8321(pUC:EAST1/K88ac), 8322(pBR:EAST1/K88ac), or strain 8323(pBelo:EAST1/K88ac) was used in RT-PCR and quantitative PCR. Quantitative PCR showed the ΔCT values of the amplified astA gene from strains 17-2 and 8722 (17-2/K88ac) were 20.6±0.01 and 20.8±0.30, with no significant differences (p=0.64). The ΔCT values of the astA gene from strains 8321, 8322, 8323 and 8231(-) were 12.3±0.02, 14.1±0.04, 17.3±0.11 and 32.7±0.66, respectively. That showed that yields of the astA gene amplicons from strains 8321, 8322, 8323 were correlated accordingly to the astA-gene plasmid copy numbers in these strains.

EAST1 was Detected in Recombinant Strains via Competitive ELISA

EAST1 protein was detected in the overnight growth supernatant of strains 17-2, 8722, 8321 and 8322, but not from strain 8323 and the negative control strain 8231 (Fig. 1). Relative binding of anti-EAST1 antiserum to coated EAST1-ovalbumin conjugates in the wells which had culture growth supernatant of 17-2, 8722, 8321 and 8322 added was 80.7±6.4, 69.7±4.7, 51.7±11.5, and 92.3±2.5 (%), respectively. That indicated the EAST1 protein expressed by 17-2, 8722, 8321 and 8322 bound to approximately 20, 30, 48, and 8% of anti-EAST1 antiserum, respectively, and suggested the EAST1 proteins were produced and secreted by these recombinant strains.

EAST1 Recombinant Strains did not Stimulate an Increasing of Intracellular cGMP or cAMP Levels in vitro

Incubation with overnight grown culture supernatant with equal amounts of 17-2, 8722, 8321 8322, or 8323 cells did not stimulate an increasing of intracellular cGMP or cAMP levels in T-84 cells (Fig. 2). The cGMP concentrations in T-84 cells incubated with supernatants of 17-2, 8722, 8321 8322, and 8323 were 2.45±1.59, 1.64±0.44, 2.33±0.67, 1.99±0.86, and 2.24±0.20 (pmole/ml), respectively. These cGMP levels were not significantly different compared to the cGMP concentration in the T-84 cells incubated with cell culture medium (2.35±0.28; p>0.05), but were

Figure 1. EAST1 competitive ELISA to detect EAST1 protein expressed from the recombinant strains. 10 ng EAST1-ovalbumin conjugates were coated at each well of a microtiter plate at 37°C overnight. 75 μl supernatants of overnight grown culture, from an equal amount of cells, of each strain, and 75 μl of chicken anti-EAST1 serum (1:1000; GenWay Biotech, Inc., CA) was mixed and added to each well. HRP-conjugated donkey anti-chicken IgY (12500; GenWay Biotech, Inc., CA) was used as the secondary antibody. OD values were measured at 405 nm after 20 min incubation with peroxidase substrate. doi:10.1371/journal.pone.0043203.g001
significantly different compared to the cGMP in cells incubated with 2 ng STa (41.5 ± 6.36; p < 0.05) (Fig 2A). Data from the cAMP ELISA showed the cAMP concentrations in T-84 cells incubated with supernatants of strains 17-2, 8722, 8321, 8322, and 8323 were 1.23 ± 0.11, 1.32 ± 0.12, 1.27 ± 0.05, 1.30 ± 0.07, and 1.20 ± 0.07 (pmole/ml), respectively. These observed levels were not significantly different from the cAMP concentration in T-84 cells incubated with cell culture medium alone (1.04 ± 0.08; p > 0.05), but were significantly different from cAMP levels in T-84 cells incubated with 10 ng CT toxin (15.15 ± 2.62; p < 0.05) (Fig. 2B). Similarly to T-84 cells, IPEC-J2 cells showed no increases of cGMP or cAMP levels after incubation with overnight growth supernatants of strains 17-2, 8722, 8321, 8322, and 8323. EAST1 expressed by the strains 8321 and 8322 did not cause significant short-circuit current (Isc) changes in Ussing chamber assay. Five independent Ussing chamber assay studies, 2 using IPEC-J2 cells and 3 with T-84 cells, were carried out. Data from these studies showed that the Isc changes were not significantly different in cells incubated with culture supernatants of 8322, 8321, or 8323 than that of 4AA culture medium. Among 5 studies, one study showed that incubation of culture growth supernatant of strains 8321 and 8322 caused Isc changes in T-84 cells and IPEC-J2 cells (Fig. 3A, 3B). But final analyses of 5 independent data sets showed Isc changes caused by strains 8321 and 8322 were not significant from that by 4AA culture medium alone (p = 0.08, p = 0.06) (Fig. 3C).

Figure 2. EIA ELISAs to measure stimulation of intracellular cyclic GMP and AMP in T-84 cells. Supernatants of overnight grown culture (with an equal amount of cells) from each constructed strain were incubated with 1–2 × 10⁵ T-84 cells. Panel A: Intracellular cGMP levels were measured using an EIA cGMP kit (Assay Designs), with 2 ng purified STa (from Robertson laboratory) was used as a positive control. Panel B: Intracellular cAMP levels were measured using an EIA cAMP kit with 10 ng CT as a positive control.

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Five-day Old Gnotobiotic Piglets did not Develop Diarrhea during 72 h Post-inoculation with 8722 and 8321 Strains

Strain 8321, that had the EAST1 gene expressed in a high-copy number expression vector pUC19, was used first to challenge 5-day old gnotobiotic piglets. If piglets challenged with strain 8321 developed diarrhea, then strains 8322 and 8323 that have the EAST1 gene expressed in medium- (pBR322) and low-copy (pBelo-BAC11) vectors would be used in subsequent challenge studies. After being orally challenged with $3 \times 10^9$ CFUs of strain 8722 or 8321, piglets remained healthy and showed no signs of diarrhea or dehydration during 72 h post-inoculation. Analyses of blood samples collected before and after inoculation showed total proteins decreased after inoculation, suggesting no dehydration occurred among inoculated piglets. To ensure that the negative outcome results were not caused by natural resistance of challenged piglets to ETEC, i.e. the lack of diarrhea in challenged piglets was resulted from their naturally resistance to ETEC colonization, we examined expression of K88ac receptors in each piglet. Bacterial adherence assays showed the brush border vesicles extracted from ileal tissue of the challenged piglets were bound by the K88ac fimbrial E. coli strain 8231 (16–27 bacteria per brush border vesicle). In addition, data from the quantitative colonization study showed challenged piglets were well colonized. Piglets challenged with strains 8722 and 8321 had $4.4 \pm 1.3$ and $8.5 \pm 4.0$ ($\times 10^8$) CFU bacteria colonized per gram of ileal tissue at 72 h post-inoculation. Differences of colonization in piglets inoculated with these two strains were statistically not significant ($p = 0.11$).

Colony screening indicated challenge strains had the introduced plasmids retained, as all 10 colonies (of each piglet) transferred from the LB plates grew well on ampicillin-supplemented (100 µg/ml) plates. PCR screening proved that all colonies were positive of the astA gene. In addition, microscopic images of ileal tissue section showed bacteria colonized at small intestinal epithelial cells of the challenged piglets (Fig. 4).

**EAST1 did not Enhance LT or STa Toxin of ETEC in Stimulation of Intracellular cAMP or cGMP In vitro**

EAST1 did not act synergistically to LT or STa toxin in stimulating intracellular cAMP or cGMP in T-84 cells (Table 2). Supernatant from LB and 4AA overnight grown culture from strains 8724 (EAST1/LT) and 8607 (LT) were examined for stimulation of cAMP; whereas supernatants from 8725 (EAST1/STa) and 8295 (STa) were comparatively studied for stimulation of cGMP (Table 2). As variations were observed among studies, the test of means was used for the final analysis of all data sets. Results indicated the cGMP concentrations of T-84 cells incubated with STa alone (strain 8295) and STa/EAST1 (strain 8725) were not significantly different ($p = 0.28$). Similarly, no significant differences were detected in cAMP levels in T-84 cells incubated with EAST1 alone (strain 17-2) showed no differences compared to those incubated with only cell culture medium.
Expression of LT in strains 8607 (LT) and 8724 (LT/EAST1) was verified in a GM1 ELISA as described previously [6,10], and expression of STa toxin from strains 8295 (STa) and 8725 (STa/EAST1) was examined in a STa competitive ELISA [10,41].

**Figure 4. A microscopic image of piglet ileal section to show colonization of *E. coli* strain 8321 that expresses EAST1 toxin.** Ileal segments of piglets challenged with strain 8321 were collected at necropsy and fixed at 10% neutral buffered Formalin. Fixed tissues were embedded, sectioned, H&E stained, and examined microscopically using an Olympus IX70 inverted microscope. doi:10.1371/journal.pone.0043203.g004

Expression of LT in strains 8607 (LT) and 8724 (LT/EAST1) was verified in a GM1 ELISA as described previously [6,10], and expression of STa toxin from strains 8295 (STa) and 8725 (STa/EAST1) was examined in a STa competitive ELISA [10,41].

**Table 2.** Intracellular cyclic AMP and GMP levels (pmole/ml) in T-84 cells incubated with culture supernatant of strains 8724 (EAST1/LT), 8607 (LT), 8725 (EAST1/STa), 8295 (STa), 17-2 (EAST1), or cell culture medium, measured using cAMP or cGMP EIA kits (Assay Design).

|                | cyclic AMP (pmole/ml) | cyclic GMP (pmole/ml) |
|----------------|-----------------------|-----------------------|
|                | 8724 (EAST1/LT)       | 8607 (LT)             | medium | 17-2 (EAST1) | 8725 (EAST1/STa) | 8295 (STa) | medium | 17-2 (ESAT1) |
| LB             | 280, 240*             | 300, 300*             | 2.7, 2.8 | ND           | 450, 465*         | 850, 940* | 3.6, 0.7 | ND           |
| 9.8, 10.1      | 11.7, 11.5            | 1.0, 0.9              | ND       | 99, 56       | 86,98             | 1.1, 0.9  | ND       |
| 12.6, 13.8     | 20, 14                | 1.6, 1.8              | ND       | 175, 133     | 150, 150          | 0.8, 0.9  | ND       |
| 4AA            | 10.1, 11.4            | 9.9, 9.4              | 1.0, 1.0 | 12, 1.2      | 70, 70            | 56, 52    | 3.2, 2.4 | ND           |
| 11.8, 11.8     | 12.5, 12              | 1.3, 1.2              | 1.3, 1.2 | 19, 26       | 72, 64            | 1.7, 0.7  | 0.7, 2.5 |
| 19.5, 17.5     | 10.6, 12.2            | 0.8, 1.0              | 1.2, 1.2 | 10.2, 10     | 16.5, 10.1        | 1.1, 1.1  | ND       |
|               |                       |                       |          | 46, 37       | 41, 40            | 2.6, 2.2  | 3.6, 1.2 |

*A non-acetylated version analysis was used to measure cAMP or cGMP concentrations in initial assays. ND indicates not done.

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GM1 and STa ELISAs, 400 ng GM1 (Sigma) was coated to each well of a MaxSorb plate (Nunc, Roskilde, Denmark) and 1.25 ng STa-ovalbumin conjugates (Robertson Laboratory) to each well of a Costar plate (Corning, Inc., Corning, NY), with anti-CT (1:3500; Sigma) and anti-STa (1:10,000; Robertson laboratory) as the primary antibody, respectively. HRP-conjugated goat-anti-rabbit IgG (1:2500 in GM1 and 1:10,000 in STa ELISA) was used as the secondary antibody. GM1 ELISA data showed supernatants of overnight cultures from strains 8607 and 8724 had OD readings of 0.972 ± 0.03 and 0.92 ± 0.04 (p = 0.10), respectively; and the competitive STa ELISA showed the OD values for strains 8295 and 8725 were 0.177 ± 0.02 and 0.21 ± 0.01 (p = 0.30), respectively. These ELISA data indicated that strains 8607 (LT) and 8724 (EAST1/LT) expressed an equivalent level of LT toxin, whereas strains 8725/EAST1/STa and 8925 (STa) expressed a similar level of STa toxin. Therefore, if EAST1 had synergistic effects, cells incubated with 8724 would have had a higher cAMP level, and cells incubated with 8725 would have had a higher cGMP level.

Discussion

Significance of EAST1 in diarrhea has not been determined previously. EAST1 was reported as the only toxin produced by E. coli strain O166 that caused an outbreak of gastroenteritis in Japan in 1996 [44], and EAST1-positive EAEC strain 17-2 that was suggested to associate with an outbreak in Chile [15]. However, EAST1 was also detected in EAEC and other types of E. coli strains isolated from asymptomatic or healthy individuals [15,25]. Moreover, human volunteers inoculated with 17-2 strain did not develop diarrhea [45], that suggests EAST1 is not the sole mediator to cause human diarrhea. Similar to the occurrence in human strains, the EAST1 gene was detected among ETEC isolates from pigs with diarrhea [5,6,30], but also commonly found in E. coli strains isolated from healthy pigs [28]. One study reported that one-day old gnotobiotic pigs, after oral inoculation of 1–2 × 10^10 CFUs of strain 17-2, 2 of 6 inoculated pigs died in 18 h, another 2 became severely ill, but the other 2 remained healthy [32]. It was unknown what caused such great variation in disease outcomes among these experimental pigs. The one-day old pigs, however, especially being inoculated with such a high dose, could be overwhelmed and develop diarrhea, even with an avirulent E. coli strain (D. Francis, personal communication). In the current study, we challenged 5-day-old pigs with 3 × 10^9 CFUs of inoculum. We believe that 5-day-old pigs tolerated E. coli inoculation, especially at a reasonable dose, better than the newborn pigs, and could be natural in disease progression after infection.

In order to eliminate ambiguity caused by host genetic resistance, we used the K88ac fimbrial strains 8722 (17-2/K88; not 17-2) and 8321 (EAST1/K88); and K88ac-receptor positive pigs in the pig challenge study. It has been demonstrated that experimental pigs would not develop disease even if being inoculated with a diarrheal ETEC strain if they lack receptors (at epithelial cells) to be attached by bacteria fimbriae or adhesins [46,47]. Using K88ac-receptor-positive pigs in the challenge study should generate conclusive results, because these pigs are naturally susceptible to K88ac fimbrial ETEC strains and always develop diarrhea and/or dehydration after inoculation. Data from bacterial adherence assays indicated the challenged pigs were K88ac-receptor positive. In addition, the quantitative colonization study and the microscopic study revealed pigs were well colonized by the challenge strains at small intestines. To also ensure plasmids carrying EAST1 were retained by the challenge strain 8321 in vivo, we cultured bacteria recovered from pig small intestines on LB plates and transferred 10 colonies to grow at ampicillin-supplemented plates. Data showed all colonies grew on ampicillin plates, indicating these colonies carried the plasmids that carry ampicillin resistance gene. When examined in PCR, these colonies were positive of the EAST1 gene. Future studies to culture bacteria that are isolated from ileal segments of challenged pigs simultaneously at the LB plates and antibiotics-supplemented plates (with great sampling sizes) will help us to better assess colonization of challenge strains in vivo. When we cultured 8321 at the LB and ampicillin-supplemented plates in vitro, we observed no plasmid loss occurred through three passages (2.28 ± 0.39 × 10^7 CFUs on LB vs. 2.34 ± 0.14 × 10^7 CFUs on ampicillin plates; p = 0.19). These data suggested that the EAST1 plasmids likely were stably maintained in challenge strain 8321 in vivo and in vitro. Therefore, if EAST1 was a virulence determinant in diarrhea, these challenged pigs would have developed clinic disease. Given that none of them developed any sign of diarrhea, the same as the pigs from the unchallenged negative control group, we concluded that EAST1 is unlikely a virulence determinant in porcine neonatal diarrhea. Although a positive control group was not directly included, 2 pigs from the same litter that were used in a separated project developed severe diarrhea and showed dehydration signs including backbone very prominent, skin turgescent and eyes sunken in orbits, after 24 h post-inoculation with porcine ETEC strain 3030-2.

To ensure expression of the additional K88ac fimbria did not negatively affect the expression of EAST1 in strain 8722 (17-2/K88), we conducted quantitative PCR to measure astA gene at the mRNA level and performed a competitive ELISA to determine EAST1 expression in strains 8722 and 17-2. Results showed nearly identical ACT values (20.6 ± 0.01 and 20.8 ± 0.30; p = 0.64) from amplified EAST1 gene were obtained from quantitative PCR, and similar production of EAST1 (p = 0.08) was detected in EAST1 competitive ELISA. These data indicated expression of K88ac fimbriae did not significantly affect strain 17-2 in production of EAST1. Therefore, pigs challenged with 8722 strain would not develop diarrheal disease had they been challenged with the wild-type 17-2 strain, even were these pigs well colonized by 17-2 at the small intestines. We need to point out that we verified the cloned EAST1 gene was expressed and the EAST1 proteins were produced and secreted by the constructed strains in vitro, we were unable to verify that expression of EAST1 in the challenge strains was not affected in vivo, especially considering culture conditions and prolonged incubation inside the piglet guts; even though the same host strain and the same expression vectors were demonstrated to express LT and other heat-stable toxins including STa and STb equally effective in culture medium and pig small intestines [9,48]. Unfortunately, we currently do not have optimal methods to confirm that the challenge strains express EAST1 at a similar level in vivo and in vitro. Future studies to develop methods to measure expression of EAST1 inside piglet guts, or to use an EAST1 knockout mutant strain derived from a porcine diarrheagenic E. coli strain that expresses EAST1 as the only toxin, were possible, in piglet challenge studies, will further conclusively assess the role of EAST1 in porcine diarrhea disease.

It was reported that E. coli strains carry different copy numbers of the astA gene, and thus it was suggested the gene copy numbers may affect significance of EAST1 in disease [3,23,49]. Attempting to determine whether EAST1 production influences virulence, we cloned the astA gene in three different expression vectors: the single-copy pBelo-BAC11, the medium-copy pBR322, and the high-copy pUC19 and constructed strains to express astA mRNA and protein at the levels correlated to the copy numbers of the
EAST1 does not cause diarrhea in pigs

The EAST1-positive EAEC prototype strain O42 caused diarrhea in majority of human volunteers [45]. That likely contributes to the assumption that EAST1-positive strain 17-2 could also cause diarrhea and EAST1 could be a virulence determinant in diarrhea. However, recent whole-genome sequencing data showed strain O42 carries other genes that strain 17-2 does not, such as the shETBA gene, as well as chloramphenicol and tetracycline resistance genes [53]. In an attempt to determine whether ShETBA toxin stimulates fluid secretion and causes diarrhea, we cloned the shETBA gene into vector pBR322 and expressed it in TOP 10 E. coli cells, incubated T-84 cells with supernatants of the ShETBA recombinant strain, and measured cAMP and cGMP levels with ELISAs. We found no increasing of cAMP or cGMP levels in the T-84 cells under our conditions; inferring that ShETBA toxin may also not play a significant role in diarrhea. However, we only verified the cloned gene with DNA sequencing but not the expression or secretion of the ShETBA toxin due to lack of anti-ShETBA antibodies, neither did we conduct any animal challenge studies. Observation regarding significance of ShET toxin obtained from this study could be premature. It was also found that the astA genes carried by strains O42 and 17-2 differ at nucleotides coding the 21st amino acid. This mutation, from a more hydrophobic nonpolar alanine in strain O42 to a less hydrophobic but polar threonine in strain 17-2, was speculated to reduce EAST1 toxicity and thus virulence in pigs.

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Author Contributions

Conceived and designed the experiments: WZ DCR. Performed the experiments: XR BDS SSC WZ. Analyzed the data: XR WZ. Contributed reagents/materials/analysis tools: DCR SSC BDS. Wrote the paper: WZ SSC.

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Purified EAST1 or culture ultrafiltrate was reported previously to cause short-circuit current changes to cell lines [36] and rabbit ileal tissue [35], but data from this study revealed differently. In this study, we used culture supernatant resulted from ultracentrifugation, which has EAST1 detected in competitive ELISA, were used to treat IPEC-J2 or T-84 cells. Pooled data (from 5 data sets) showed that the differences in Lc changes caused by strains 8321 and 8322 were not significant, even compared to those in cells incubated with the cell culture medium alone.

EAST1 is often compared to STa toxin due to similarity of a peptide domain [36], and was suspected to have similar biological functions [36,50]. However, unlike STa, EAST1 does not stimulate fluid accumulation in suckling mice or cause diarrhea in human volunteers [45]. Although EAST1+ EAEC strains were linked to diarrhea, it is thought that not one set of virulence factors, but rather combinations of multiple virulence factors, are associated with diarrhea [12]. Data from this study showed that EAST1 did not stimulate an increase of intracellular cGMP or cAMP. In addition, data also showed EAST1 had no synergistic effect to LT or STa toxin of ETEC in stimulation of intracellular cAMP or cGMP in vitro. Since an earlier report that indicated porcine ETEC strains tend to acquire additional toxins over time [29], and the fact that EAST1 is frequently carried along with LT, STa and STb toxins by ETEC strains associated with diarrhea in pigs [5,6,28,30], EAST1 has been speculated to have synergistic enterotoxicity in ETEC strains. Given ETEC strains expressing LT, STa, or sometimes STb alone are already sufficiently virulent to cause diarrhea in pigs [7,9,10], we questioned whether acquisition of an additional toxin makes these ETEC strains more virulent, possibly to overcome host immunity gained from natural exposure to ETEC pathogens, vaccination or antibiotic treatment. Data from this study clearly indicated that EAST1 did not enhance LT in stimulation of intracellular cAMP or STa in stimulation of intracellular cGMP in the human cell line T-84. Therefore, EAST1 is unlikely to be a synergistic factor to other ETEC toxins in stimulation of fluid hyper-secretion. It was also reported that EAST1 positive E. coli, mainly EAEC strains, cause intestinal inflammation [51,52]. But histological examination found no apparent inflammation occurring in small intestines of the challenged piglets, suggesting EAST1 alone may not contribute significantly to intestinal inflammation. However, future studies to examine IL-8 or other cytokine levels in pigs challenged with strain 8321 that had EAST1 cloned and expressed in a medium-copy or a single-copy vector, or perhaps EAST1-positive E. coli field isolates that carry one or a few copies of the astA gene, would cause diarrhea in pigs.
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