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Distribution and Molecular Characterization of Enteroaggregative *Escherichia coli* Isolated from Children in A Case-control Study of Acute Diarrhea in Thailand

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Abstract

Background: Enteroaggregative *Escherichia coli* (EAEC) is a major cause of acute diarrhea in children, adults, and travelers as well as an important cause of persistent diarrhea. Due to the limited information on EAEC virulence genes, EAEC isolates isolated from children with diarrhea and asymptomatic controls in Thailand were characterized for the presence of virulence factors.

Method: A study of diarrhea etiology in children from Thailand was conducted during 2008-2009 and 301 EAEC isolates were identified by pCVD432 probe/hybridization assays from diarrhea cases and controls. A total of 200 EAEC isolates were further characterized by PCR for EAEC genotypes, aggregative adherence fimbria (AAF/I-AAF/IV), serine protease autotransporters of Enterobacteriaceae (SPATEs) class I & II, and enterotoxin genes.

Results: The prevalence of EAEC isolates in this study were 7.7% and 9.1% among cases (n=1803) and controls (n=1790). Of 200 EAEC isolates, 69% of case and 58% of control isolates were classified as typical EAEC genotype. AAF/I (aggA) was the major fimbria type for cases (37%) and controls (23%), followed by AAF/III (9%, 13%), AAF/II (11%, 17%) and AAF/IV (5%, 3%). In confirmed cases, class II SPATE-sepA (19%, 7%) and pic (75%, 60%) and enterotoxin-set (75%, 61%) genes were detected in significantly more strains when compared to the controls. Additionally, typical EAEC isolates containing genes for AAF/I, Class II SPATEs (sepA, pic), and enterotoxin (set) were detected more frequently in cases than controls (P value<0.05).

Conclusion: This study suggests virulence gene heterogeneity of EAEC isolated from children with diarrhea as compared to asymptomatic controls, and that specific virulence genes are associated with diarrhea.

Keywords: Virulence genes; Aggregative adherence; Fimbria; SPATEs; Toxin; Diarrhea; Enteroaggregative *E. coli*; EAEC

Introduction

Enteroaggregative *E. coli* (EAEC) is a significant cause of acute gastroenteritis in both developing and industrialized countries [1-3]. EAEC is associated with traveler's diarrhea [4,5], endemic chronic pediatric diarrhea [6,7] and persistent diarrhea in immunocompromised patients [8,9]. EAEC is increasingly considered as an emerging enteric pathogen [10,11]. Among the diarrheagenic *E. coli* pathotypes, EAEC is more heterogeneous, with limited information on the roles and importance of specific virulence genes and putative gene products.

EAEC was originally defined by a phenotypic pattern described as aggregative adherence (AA) to HEp-2 cells [12]. Studies on the mechanisms involved in pathogenesis of EAEC suggested the presence of a variable package of co-inherited virulence genes carried on chromosomal islands and virulence plasmids [13]. Specific genes identified included a master transcriptional regulator (AggR) encoded on an EAEC virulence plasmid, pAA [14]. A cluster of aggR-activated

genes (AAI) responsible for encoding type IV secretion was also found on a chromosomal island [15]. EAEC strains carrying multiple elements of the AggR regulon, and the genes encoding aatA, aggR, and aaiC are considered "typical" EAEC, even though their pathogenicity potential remains unknown.

A cryptic DNA sequence termed 'CVD432' was 1) associated with aggregative adherence, 2) present on the plasmid pAA at locus aat, and 3) used as a molecular marker for EAEC in prior epidemiologic studies [2,16]. However, not all phenotypic EAEC strains are positive for CVD432, suggesting that additional virulence factors may be involved with pathogenesis [17].

The mechanisms of EAEC pathogenesis were reported as 1) adherence to the intestinal mucosa, 2) expression and release of enterotoxins and cytotoxins, and 3) subsequent host mucosal inflammation [18-20]. Adherence is mediated in part by aggregative adherence fimbriae (AAF) variants I-IV [21-25]. As many EAEC strains lack a known fimbria, other adherence mechanisms are likely present. EAEC toxins include serine protease autotransporters of the Enterobacteriaceae (SPATEs) [26,27]. The SPATEs pic and sepA were linked to EAEC pathogenesis [28,29]. Other toxins, to include Shigella

enterotoxin (ShET1) and heat stable toxin (EAST-1), were also associated with EAEC diarrhea [30,31].

In Southeast Asia, EAEC is commonly isolated from stool specimens collected from both diarrhea cases and non-diarrhea controls, thus obscuring its contribution to diarrhea. This study determined the prevalence of EAEC and characterized EAEC isolates for adherence fimbria, enterotoxins, SPATE genes, and their significance to diarrhea cases and non-diarrhea controls from children in Thailand.

Materials and Methods

Study site and EAEC isolation and identification

A diarrheal surveillance study was conducted by the Department of Enteric Diseases, AFRIMS, Bangkok, Thailand and the Department of Medical Sciences, Ministry of Public Health, Thailand from 2008-2009 to identify diarrhea etiologic agents, including bacteria, parasites, and viruses, in children with and without diarrhea. The study was approved by the ethical review committee for Research in Human Subjects, Ministry of Public Health (MOPH), Nonthaburi, Thailand and the Walter Reed Army Institute of Research (WRAIR) IRB, Silver Spring, MD, USA . The study included 1803 children with diarrhea and 1790 non-diarrhea controls, enrolled with parental consent at different provincial hospitals throughout Thailand. At the time of study, stool samples were cultured to identify bacterial enteric pathogens (*Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter* spp., *Aeromonas* spp., *Pleisiomonas* spp., and *E. coli*) by standard microbiology techniques [32]. ELISA assays were used for the detection of rotavirus, adenovirus, and astrovirus (R-Biopharm AG, Darmstadt, Germany), and *Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium* (ProspecT, Remel Products, Lexena, KS, USA) according to the manufacturer's instructions. Norovirus was detected by real-time PCR assay. Five lactose-fermenting and/or five non-lactose fermenting *E. coli* from each stool sample, identified on MacConkey agar plates, were spotted on a nylon membrane and processed for DNA hybridization using nonradioactive digoxigenin-labeled probe from pCVD432 [16] for the identification of EAEC. All *E. coli* isolates positive by CVD432 probe-hybridization assays were archived at -70°C for further studies.

EAEC isolates for characterization

A total of 301 EAEC isolates were identified and archived from the diarrheal surveillance study described above. Two-hundred archived EAEC isolates, 100 from cases and 100 from controls, were selected for further molecular characterization. Of the 100 selected EAEC isolates from cases, 29 isolates were from stool samples where EAEC was detected as a single pathogen and 71 isolates were from co-infections. Of the 100 selected EAEC isolates from controls, 50 isolates were from stool samples where EAEC was detected as a single pathogen and 50 were from co-infections.

Genomic DNA extraction of EAEC isolates

Frozen EAEC isolates were freshly grown on Tryptic Soy Agar (TSA) plates. A suspension of bacteria in deionized water (molecular grade) was boiled at 100°C for 20 min and centrifuged at 10,000 rpm for 10 min., and the resulting supernatant was further used as DNA template for further studies.

PCR assays for EAEC genotype, adherence fimbria and virulence genes

For genotype identification, PCR using specific primers to confirm EAEC genotypes, a transcriptional activator gene (aggR), and chromosomal locus gene (aaiC) with DNA templates from selected EAEC isolates was conducted, as previously described [15]. For detection of EAEC aggregative adherence fimbria types, specific primers for the aggA, aafA, agg3A and agg4A genes were used to determine AAF fimbria type I to IV, respectively, as previously described [22,28,33]. For EAEC virulence-related genes, specific primers for Class I SPATEs genes (sat, sigA, and pet), Class II SPATEs genes (pic and sepA), and two enterotoxin genes (astA and set) genes were also included, as previously described [22,28,33].

Multiplex PCR reaction was performed for detection of: 1) sepA, pic, and sigA 2) pic and pet; and 3) aaiC and astA, via three separate assays whereas singleplex PCR reactions were used to amplify and detect: 1) aggR 2) 4 fimbria genes aggA, aafA, agg3A, and aag4A 3) the SPATE gene sat; 4) and the enterotoxin gene set in this study. Positive controls were EAEC type strains 17-2, EAEC 042, and *Shigella flexneri* 2457T. *E. coli* ATCC 11175 was used as a negative control. Resulting amplified products were analyzed by gel electrophoresis and ethidium bromide staining. A 2-Log DNA ladder and a 100bp-DNA ladder (New England BioLabs Inc., MA, USA and Gene Ruler., Thermo Fisher Scientific, MA, USA) were used as standard markers.

Data analysis

The frequency of each gene was calculated as numbers and percentages detected in EAEC isolates from cases and controls. The association of each gene with case or control status was tested for significant differences by Fisher's exact test-two tailed using GraphPad QuickCalcs Web site: http://www.graphpad.com/quickcalcs/contingency1.cfm (accessed February 2017). P values ≤ 0.05 were considered statistically significance.

Results

Identification and prevalence of EAEC isolates among cases and controls

The prevalence of EAEC by the CVD432 probe-hybridization assay was 7.7% (139/1803) and 9.1% (162/1790) from cases and controls, respectively. EAEC was isolated both as a single pathogen and as part of a co-infection in stool from both cases and asymptomatic controls. Among EAEC isolates from cases, 20.9% (29/139) were detected as a single pathogen and 79.1% (110/139) were isolated from stool samples with mixed infections (Table 1). In contrast, EAEC isolated from control stool samples as a single pathogen and mixed pathogens were roughly equal (cases: 50.6%; controls: 49.4%) respectively. EAEC was detected most often with rotavirus, *Salmonella* spp., *Campylobacter* spp., and norovirus in co-infections; however, EAEC among controls was detected most often with atypical enteropathogenic *E. coli* (only eae gene positive), *Aeromonas* spp., *Salmonella* spp. and *Campylobacter* spp. in co-infections. A total of 200 EAEC isolates, 100 from cases and 100 from controls, were selected for further molecular characterization.

Number of samples	Number of EAEC isolates	Number of EAEC isolates	Number of EAEC isolates characterized

	positive by pCVD432 probes (%)	Single pathogen	Mixed pathogen	Single pathogen	Mixed pathogen
Cases (1803)	139 (7.7)	29	110	29	71
Controls (1790)	162 (9.1)	82	80	50	50

Table 1: Prevalence of EAEC isolated from stool of childhood diarrhea cases or asymptomatic controls in Thailand 2008-2009.

Detection of EAEC genotypes

Figure 1 depicts an ethidium bromide stained gel electrophoresis image of PCR generated amplicons from a series of multiplex PCR and singleplex PCR reactions, including positive controls for reference. Of the 200 EAEC isolates, the aatA, aggR, and aaiC triple combination genotype was the most commonly detected at 69% and 58% from cases and controls, respectively.
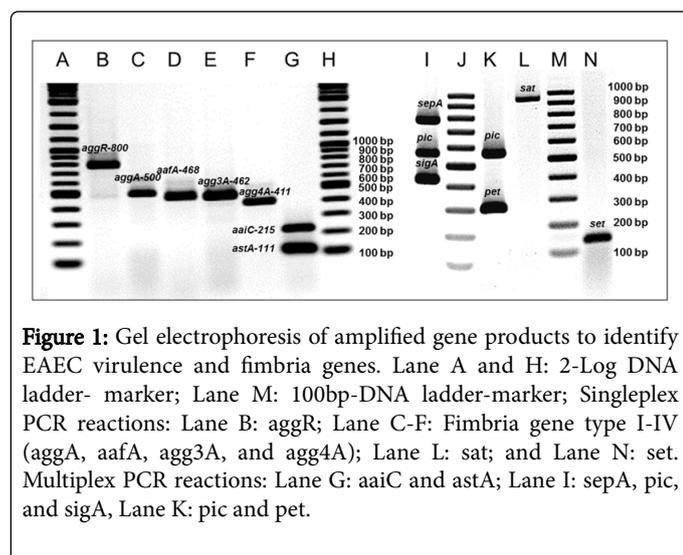

Figure 1: Gel electrophoresis of amplified gene products to identify EAEC virulence and fimbria genes. Lane A and H: 2-Log DNA ladder- marker; Lane M: 100bp-DNA ladder-marker; Singleplex PCR reactions: Lane B: aggR; Lane C-F: Fimbria gene type I-IV (aggA, aafA, agg3A, and agg4A); Lane L: sat; and Lane N: set. Multiplex PCR reactions: Lane G: aaiC and astA; Lane I: sepA, pic, and sigA, Lane K: pic and pet.

The EAEC double genotype aatA, aaiC, and single genotypes aatA or aggR, were found less frequently. However, only 8% of the cases and 8% of the control EAEC isolates were positive for aatA. The EAEC genotype aatA, aggR, was detected significantly more often in the controls (26%) when compared to the cases (12%; P=0.018), whereas the frequency of other EAEC genotypes were not significantly different (Table 2).

Detection of adherence fimbria genes /types

AggA, encoding for adherence fimbria gene type I (AAF/I), was detected in 37% and 23% of the cases and controls, respectively, and was the major fimbria type among all EAEC isolates (P=0.044; Table 2). The aafA, agg3A and agg4A genes, encoding for AAF/II, AAF/III and AAF/IV respectively, were detected less commonly and did not show significant differences between EAEC isolates from cases and controls.

Detection of enterotoxin and SPATEs genes

Two enterotoxin genes, astA and set, encoding for heat-stable toxin (EAST-1) and *Shigella* enterotoxin respectively, were commonly

detected in EAEC isolates in this study (Table 2). There was no significant difference in the presence of astA in cases and controls, and set was detected significantly more often in cases (75%) as compared to the controls (66%; P=0.048).

Genes	Target	Prevalence of genes (%)		
		Cases (n=100)	Controls (n=100)	P value (Fisher's exact test, two-sided)
EAEC genotype				
aatA	CVD432 positive	8	8	NS
aatA, aggR	Transcriptional activators gene encoded for the expression of fimbria	12	26	0.018
aatA, aaiC	Secreted protein	11	8	NS
aatA, aggR, aaiC	Typical EAEC traits	69	58	NS
Aggregative adherence fimbria				
aggA	Aggregative adherence fimbria I (AAF/I)	37	23	0.044
aafA	Aggregative adherence fimbria II (AAF/II)	9	13	NS
agg3A	Aggregative adherence fimbria III (AAF/III)	11	17	NS
agg4A	Aggregative adherence fimbria IV (AAF/IV)	5	3	NS
Class I SPATE				
sat	Secreted autotransporter toxin	30	28	NS
sigA	IgA protease-like homolog	1	0	NS
pet	Plasmid-encoded toxin	13	14	NS
Class II SPATE				
sepA	Shigella extracellular protease	19	7	0.019
pic	Serine protease precursor	75	60	0.034
Enterotoxin				
astA	heat-stable toxin (EAST-1)	28	33	NS
set	Shigella enterotoxin 1	75	61	0.048
NS: Not Significant				

Table 2: Molecular characteristics of EAEC genotypes and virulence traits among 200 EAEC isolates, by cases and controls.

The Class I SPATE genes, sigA and pet, were detected less frequently than sat, and there was no significant difference in the presence of these genes in cases and controls (Table 2). The class II SPATE genes, sepA and pic, were detected in 19% and 75% from cases and 7% and

60% from controls, respectively. Both sepA and pic were detected more frequently and significantly in cases than controls (P=0.019 and 0.034).

Association of adherence fimbria types, SPATEs and toxin genes among EAEC isolates

Figure 2 depicts the association of adherence fimbria type I-IV, SPATEs, and enterotoxin genes of EAEC from cases and controls. EAEC of adherence fimbria type I (AAF/I) harbored pic and set in 86% and 65% of isolates from cases and controls, respectively.
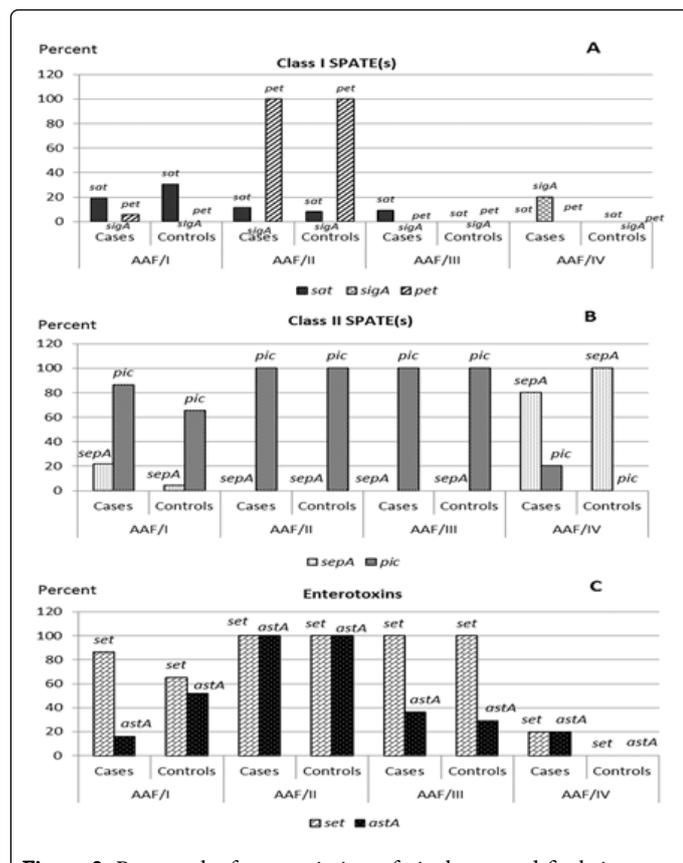

Figure 2: Bar graphs for association of virulence and fimbria genes among 200 EAEC isolates, by cases/controls. A: Percent of class I SPATE (sat, pet, and sigA) and fimbria types (AAF/I-IV) of 200 EAEC isolates, B: Percent of class II SPATE (pic and sepA) and fimbria types (AAF/I-IV) of 200 EAEC isolates, C: Percent of enterotoxins (set and astA) and fimbria types (AAF/I-IV) of 200 EAEC isolates.

In contrast, all EAEC isolates with fimbria type II (AAF/II) carried genes encoding for SPATEs (pet, pic) and enterotoxin (set, astA) in both cases and controls. All EAEC isolates with fimbria type III (AAF/III) harbored genes encoding for pic and set in 100% of isolates from cases and controls, but the SPATEs (sat, pet, and sepA) and enterotoxin (astA) genes were less prevalent in these isolates. Most EAEC isolates positive for adherence fimbria type IV (AAF/IV) possessed sepA (80% and 100% of isolates from cases and controls, respectively), while 20% of these EAEC isolates from cases had genes encoding for the other SPATEs (sigA and pic) or enterotoxins (set and astA). None of the EAEC with fimbria type IV from controls carried class I SPATEs (sigA, pet and sat) and enterotoxins (set and astA).

Association of fimbria types, SPATEs genes and enterotoxin genes among typical EAEC isolates in cases and controls

Of the 200 EAEC isolates characterized under study, 69 isolates from cases and 58 from controls possessed typical EAEC traits. Typical EAEC isolates with fimbria type I (AAF/I) were detected more commonly in cases than controls (47.8% and 27.6%; P=0.027; Table 3). Moreover, the typical EAEC-AAF/I isolates possessing either pic or set, or all three genes set, pic and sepA, were detected significantly more often in cases than controls, respectively (P=0.026, 0.026 and 0.038). In contrast, typical EAEC with AAF/II was observed more often in controls than cases, and there was no significant association of typical EAEC genotypes with fimbria types AAF/II- IV.

EAEC traits	Cases (n=69)	Controls (n=58)	P value (Fisher's exact test, two-sided)
Typical EAEC, AAF/I	33	16	0.027
Typical EAEC, AAF/II	9	12	NS
Typical EAEC, AAF/III	11	17	NS
Typical EAEC, AAF/IV	1	0	NS
Typical EAEC, AAF/I, sepA	8	1	0.038
Typical EAEC, AAF/I, pic	32	15	0.026
Typical EAEC, AAF/I, set	32	15	0.026
Typical EAEC, AAF/I, sepA, pic, set	8	1	0.038
NS: Not significant			

Table 3: Association of typical EAEC isolates, adherence fimbria types, and serine protease autotransporters of *Enterobacteriaceae* (SPATEs) genes, by cases and controls.

Discussion

There are limited data on the prevalence of EAEC genotypes and distribution of genes for adherence fimbria, enterotoxins, and SPATEs among EAEC isolates from Southeast Asia. This study reported the prevalence of these genes in 200 EAEC isolates collected from a case-control diarrhea surveillance study from young children in Thailand. All EAEC isolates in this study were positive for pCVD 432 and then further characterized for aggR and aaiC genotypes. The typical EAEC genotype (aatA, aggR, aaiC) was the most prevalent EAEC genotype detected (>50%). However, the frequency of the typical EAEC genotype was not significantly different between cases and controls.

Only 59% (118/200) of EAEC isolates possessed genes for fimbria and we observed a varied distribution of fimbria types. The frequency of adherence fimbria type I-AAF/I (aggA) was the greatest among EAEC isolates from both cases and controls, followed by AAF/III (agg3A), AAF/II (aafA), and AAF/IV (agg4A). These findings were similar to other studies [28,29] where nearly half of clinical EAEC isolates do not possess known major fimbria subunits.

This study concluded that EAEC isolates harbored both class I & II SPATE genes, but the proportion detected differed. Guyer et al. [34] reported that sat was associated with pathogenicity of uropathogenic *E. coli* and diffuse-adhering *E. coli* (DAEC). A previous study noted that sat was detected in 74.5% of clinical EAEC isolates from Denmark [28], while this effort observed sat in only 30% and 28% of EAEC isolates from cases and controls respectively.

SepA encodes for a major extracellular protein that promotes tissue invasion in *S. flexneri* [35]. Previous studies suggested that sepA is a valuable identification marker for virulent EAEC [18,29]. This study identified less distribution of sepA in EAEC isolates from Thailand compared to previous studies [28,29]. Therefore, the contribution of sepA to EAEC virulence should not be overlooked. Pic was related to a *Shigella* mucinase originally described in EAEC, *S. flexneri* 2a, and uropathogenic *E. coli*, that mediates penetration and enhances host inflammatory responses [18,26]. Pic was significantly associated with EAEC infection as a single pathogen in prolonged diarrhea as compared to acute diarrhea in Peruvian children [36]. However, this study included only EAEC isolates with acute diarrhea, and as such, the association with prolonged diarrhea could not be confirmed. Although the prevalence of sepA was not as high as pic, this study reported a stronger association of sepA with EAEC isolates from diarrhea cases when compared to those bearing the pic gene. SetAB, encoding for *Shigella* enterotoxin ShET1, is located on a complementary strand of pic [27,33]. For this reason, both genes should be equally detected in EAEC isolates. Results from this study matched the predicted distribution of these two genes among characterized EAEC isolates.

Although there was no significant association of adherence fimbria types, SPATEs and enterotoxin genes in the 200 EAEC isolates from cases and controls in this study, there was an observed association of pet and sepA genes with EAEC isolates possessing AAF/II and AAF/IV fimbria, respectively, more commonly than with other fimbria types. This finding was similar to a previous study where the authors suggested co-transmission or functional linkage of pet and AAF/II [28]. Additionally, fimbria type V (AAF/V-agg5A) was recently reported in 12% of EAEC isolates from stool specimens of travelers in Denmark [37]; however, this study did not include PCR detection of this fimbria type.

More EAEC isolates were identified in asymptomatic controls than in cases in this study, and there was no significance association of typical EAEC isolates in samples where the organism was a mono-infection or co-infection (data not shown). This data strongly demonstrates that typical EAEC isolates, in combination with fimbria type I (AAF/I), SPATEs (sepA, pic) and enterotoxin (set) genes, were significantly associated more commonly with diarrhea cases than controls. As a result, the authors suggest that these genes are potential virulence markers for EAEC isolates, and that other host factors may serve an important role in EAEC pathogenesis. Future studies are warranted, particularly looking at the distribution of these genes in EAEC isolates in different geographic regions of the world.

Declaration of Conflict of Interest

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70-25.

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