1. Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol* Rev 1998; 62:1301-14. Review.

2. Kaper JB, Morris JG Jr, Levine MM. Cholera. *Clin Microbiol* Rev 1995; *8*: 48-86.

3. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. Comparative genomic analysis of *Vibrio cholerae* genes that correlate with cholera endemic and pandemic disease. *Proc Natl Acad Sci USA* 2002; 99 : 1556-61.

4. Keasler SP, Hall RH. Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. *Lancet* 1993; *34* : 1661.

5. Waldor MK, Rubin EJ, Pearson GDN, Kimsey H, Mekalanos JJ. Regulation, replication, and integration functions of the *Vibrio cholerae* CTXΦ are encoded by region RS2. *Mol Microbiol* 1997; *24*: 917–26.

6. Davis MB, Moyer KE, Boyd EF, Waldor MK. CTX prophages in classical biotype *Vibrio cholerae*: functional phage genes but dysfunctional phage genomes. *J Bacteriol* 2000; *182*: 6992–8.

7. Lin W, Fulner KJ, Clayton R, Sexton JA, Rogers MB, Calia KE, *et al.* Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc Natl Acad Sci USA* 1999; *96*: 1071-6.

8. Rader AE, Murphy JR. Nucleotide sequences and comparison of the hemolysin determinants of *Vibrio cholerae* El Tor RV79(Hly+) and RV79(Hly-) and classical 569B(Hly-). *Infect Immun* 1988; *56*: 1414-9.

9. Alm RA, Manning PA. Biotype-specific probe for *Vibrio cholerae* serogroup O1. *J Clin Microbiol* 1990; *28*: 823-4.

10. O'Shea WA, Finnan S, Reen FJ, Morrissey JP, O'Gara F, Boyd EF. The Vibrio seventh pandemic island-II is a 26.9 kb genomic island present in *Vibrio cholerae* El Tor and O139 serogroup isolates that shows homology to a 43.4 kb genomic island in *V. vulnificus*. *Microbiol* 2004; *150*: 4053-63.

11. Nair GB, Mukhopadhyay AK, Safa A, Takeda Y. Emerging hybrid variants of *Vibrio cholerae* O1. In: S. M. Faruque and G.B. Nair, editors. *Vibrio cholerae*: Genomics and Molecular biology. Norwich (UK): Horizon Scientific Press; In press 2008. p.179-90.

12. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. *Lancet* 2005; *63*: 223–33.

13. Finkelstein, RA. Cholera, *Vibrio cholerae* O1 and O139, and other pathogenic vibrios. [cited 2006 February 25]. Available from: http://gsbs.utmb.edu /microbook/ch024.htm.

14. Finkelstein RA, Burks F, Zupan A, Dallas WS, Jacob CO, Ludwig DS. Epitopes of the cholera family of enterotoxins. *Rev Infect Dis* 1987; *9*: 544–61.

15. Olsvik O, Wahlberg J, Petterson B, Uhlen M, Popovic T, Wachsmuth IK, *et al.* Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *J Clin Microbiol* 1993; *31*: 22–25.

16. Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* 2002; *40*: 3296–9.

17. Ansaruzzaman M, Bhuiyan NA, Nair GB, Sack DA, Lucas M, Deen JL, *et al*; the Mozambique Cholera Vaccine Demonstration Project Coordination Group. Cholera in Mozambique, variant of *Vibrio cholerae*. *Emerg Infect Dis* 2004; *10*: 2057–9.

18. Faruque SM, Tam VC, Chowdhury N, Diraphat P, Dziejman M, Heidelberg JF, *et al.* Genomic analysis of the Mozambique strain of *Vibrio cholerae* O1 reveals the origin of El Tor strains carrying classical CTX prophage. *Proc Natl Acad Sci USA* 2007; 104 : 5151-6.

19. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, *et al.* Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. *J Clin Microbiol* 2006; *44*: 4211-3.

20. Tamplin ML, Jalali R, Ahmed MK, Colwell RR. Variation in epitopes of the B subunit of *Vibrio cholerae* non-O1 and *Vibrio mimicus* cholera toxins. *Can J Microbiol* 1990; *36* : 409–13.

21. Raychoudhuri A, Patra T, Ghosh K, Ramamurthy T, Nandy RK, Takeda Y, Balakrish-Nair G, Mukhopadhyay AK. Classical ctxB in *Vibrio cholerae* O1, Kolkata, India. Emerg Infect Dis. 2009;15:131-132.

22. Raychoudhuri A, Mukhopadhyay AK, Ramamurthy T, Nandy RK, Takeda Y, Nair GB. Biotyping of *Vibrio cholerae* O1: time to redefine the scheme. Indian J Med Res. 2008; 128:695-698.

23. Morita M, Ohnishi M, Bhuiyan NA, Nusrin S, Alam M, Siddique AK, *et al.* Development and validation of a mismatch amplification mutation assay PCR to distinguish between the cholera toxin B subunit of classical and El Tor biotypes of *Vibrio cholerae* O1. *Microbiol Immunol*; 2008; 52:314-317.

24. World Health Organization. Cholera 2006. *Wkly Epidemiol Rec* 2007; *82*: 273-84.

25. Safa A, Sultana J, Cam PD, Mwansa JC, Kong RYC. Classical cholera toxin producing *Vibrio cholerae* O1 hybrid El Tor strains in Asia and Africa. *Emerg Infect Dis* 2008; *14*:987-8.

26. Cooper KL, Luey CK, Bird M, Terajima J, Nair GB, Kam KM, Arakawa E, Safa A, Cheung DT, Law CP, Watanabe H, Kubota K, Swaminathan B, Ribot EM.

Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. Foodborne Pathog Dis. 2006;3:51-8.

27. Blaser MJ, Wells JG, Feldman RA, Pollard RA, Allen JR. *Campylobacter* enteritis in the United States: a multicentre study. Ann Intern Med 1983; 98:360-5.

28. De Mol P, Brasseur D, Hemelhof W, Kalala T., Butzler JP, Vis HL. Enteropathogenic agents in children with acute diarrhoea in rural Zaire. Lancet i, 1983; 516-18.

29. Linton, D., Lawson, A.J., Owen, R.J., Stanley, J., 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. 35, 2568—2572.

30. Oberhelman RA, Tayor DN. *Campylobacter* infections in developing countries. In: Nachamkin I, Blaser JM (Eds.), *Campylobacter*, second ed. ASM Press, Washington, DC, 2000; pp. 139-154.

31. Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, Garg S, Bhattacharya SK, Nair GB, Nishibuchi M. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. J Clin Microbiol. 1997; 35:3150-55.

32. Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev. 2007; 20:39-48.

33. Matsumoto C, Okuda J, Ishibashi M, Iwanaga M, Garg P, Rammamurthy T, Wong HC, Depaola A, Kim YB, Albert MJ, Nishibuchi M. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J Clin Microbiol. 2000; 38:578-85.

34. Williams TL, Musser SM, Nordstrom JL, DePaola A, Monday SR. Identification of a protein biomarker unique to the pandemic O3:K6 clone of *Vibrio parahaemolyticus*. J Clin Microbiol. 2004; 42:1657-65.

35. Park KS, Ono T, Rokuda M, Jang MH, Okada K, Iida T, Honda T. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. Infect Immun. 2004; 72:6659-65.

36. Kam KM, Luey CK, Parsons MB, Cooper KL, Nair GB, Alam M, Islam MA, Cheung DT, Chu YW, Ramamurthy T, Pazhani GP, Bhattacharya SK, Watanabe H, Terajima J, Arakawa E, Ratchtrachenchai OA, Huttayananont S, Ribot EM, Gerner-Smidt P, Swaminathan B; *Vibrio parahaemolyticus* PulseNet PFGE

Protocol Working Group. Evaluation and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping *Vibrio parahaemolyticus:* an international multicenter collaborative study. J Clin Microbiol. 2008 46:2766-2773.

37. Chowdhury NR, Chakraborty S, Ramamurthy T, Nishibuchi M, Yamasaki S, Takeda Y, Nair GB. Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. Emerg Infect Dis. 2000; 6:631-36.

38. González-Escalona N, Martinez-Urtaza J, Romero J,' Espejo RT, Jaykus LA, DePaola A. Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. J Bacteriol. 2008; 190:2831-40.

39. Kimura B, Sekine Y, Takahashi H, Tanaka Y, Obata H, Kai A, Morozumi S, Fujii T. Multiple-locus variable-number of tandem-repeats analysis distinguishes *Vibrio parahaemolyticus* pandemic O3:K6 strains. J Microbiol Methods. 2008; 72:313-20.

40. Chowdhury NR, Stine OC, Morris JG, Nair GB. Assessment of evolution of pandemic *Vibrio parahaemolyticus* by multilocus sequence typing. J Clin Microbiol. 2004; 42:1280-82.

Title: Virulence Factors and Antimicrobial Susceptibility of Enteroaggregative *Escherichia coli* Isolated from Children

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Summary: 300~500 words:

Enteroaggregative *Escherichia coli* (EAEC) have been reported as causative agents of persistent diarrhea in infants and young children in developing countries, outbreaks of food borne illness in industrialized countries. A growing number of reports and the rising proportion of diarrheal cases in which EAEC are implicated suggest that EAEC are important emerging agents of pediatric diarrhea. In this study, EAEC strains obtained from children with diarrheic symptoms in Thailand during years 2008-2009 were characterized for virulence associated genes of EAEC by multiplex PCRs, determined for invasions property, and antimicrobial susceptibility tests by agar disk diffusion.

Determination of virulence-associated genes by PCR demonstrated that 32% of 268 EAEC strains were positive for the presence of aggregative fimbriae genes (*agg*A, *aaf*A, *agg3*A), but 68% were negative. About 23 % and 13% of EAEC strains were *astA*, and *pet* genes positive, respectively. The AAF and enterotoxin genes negative strains which accounted for 47% of EAEC were found the most frequent.

EAEC strains were randomly selected and assayed for invasion to HeLa cells. Of 55 EAEC strains tested, 10.9% were found to be highly-invasive, 12.7% were invasive, and 76.4% were non-invasive. The CFU/well of highly-invasive EAEC and invasive-EAEC strains decreased gradually from 0 to 6 h of post-infection, with a marked decrease at 24 h demonstrated the inability of EAEC to proliferate with the cells.

About 90% of 192 EAEC strains were resistant to antimicrobial drug. The frequency of resistant to 2, 3, 4, and more than 5 drugs were found in 9.8%, 17.9%, 25.4%, 27.2% and 19.7% strains of EAEC, respectively. EAEC strains were 90%, 76%, and 73% resistant to AMP, TE,

and SXT, respectively. About 7% of EAEC strains were confirmed as ESBL-producing.

These result indicated that EAEC strains in Thailand are heterogeneous with regard to their virulence factor profile. The expression of aggregative adherence (AA) phenotype can be mediated by as-yet-unidentified, alternative adherence factor(s). Toxins or factors other than enterotoxins may play a role in pathogenesis of EAEC. Some EAEC strains are invasive, but they cannot survive. The majority of EAEC are drug resistance and some are ESBL-producing that may cause severe complication to children infected with EAEC. Genotypic assay of the ESBL-producing EAEC needs to be further investigations.

Purpose:

EAEC are defined as *E. coli* strains that do not secrete heat-labile (LT) and heat-stable (ST) enterotoxins, and adhere to HEp-2 or HeLa cells in an aggregative adherence (AA) pattern. Since Nataro *et al* [1] first described EAEC as an agent of pediatric diarrhea in 1987, a growing number of studies have supported the association of EAEC with persistent diarrhea in infants and young children in developing countries. Recent outbreaks of EAEC as a cause of food borne illness in industrialized countries have been reported. In 1993, a massive outbreak of EAEC diarrhea occurred in the Gifu Prefecture, Japan, in which 2,697 children at 16 schools became ill after consuming contaminated school lunches [2]. Four outbreaks of EAEC-related diarrhea occurred in the United Kingdom in 1994 [1]. A growing number of reports and the rising proportion of diarrheal cases in which EAEC are implicated suggest that EAEC are important emerging agents of pediatric diarrhea. The pathogenesis of EAEC infection is not well understood. Histopathologic examination of tissues from infected patients and animal models have shown that EAEC strains enhance mucus secretion from the mucosa, with bacteria trapped in a bacteria-mucus biofilm. Human volunteers who ingested EAEC strains excrete mucoid stools. The role of excess mucus production in EAEC is unknown. Three types of aggregative adherence fimbriae types I (AAF/I), II (AAF/II), III (AAF/III) have been shown to be required for expression of an AA pattern on the surfaces of HEp-2 and HeLa cells [3,4,5]. In addition, a variety of other virulence factors have been described, including the heat-stable enterotoxin (EAST1) [6], and the plasmid-encoded toxin (Pet) [7]. The role of EAST1 and Pet in mucus secretion has not yet been determined.

The study of prevalence of childhood diarrhea-associated *E. coli* in Thailand, during 1996 – 2000 showed that EAEC was the pathotype most frequently isolated in Thai children in every year, indicating the important of EAEC in Thai children [8]. Little is known about the pathogenesis of EAEC in Thailand. Therefore, analysis of virulence factors of EAEC is required to better understand their role in diarrhea disease that may lead to the improvement of laboratory diagnosis, prevention and control of EAEC infections.

This study was designed to investigate the virulence factors and antimicrobial susceptibility of Enteroaggregative *Escherichia coli* (EAEC) strains isolated from children in Thailand.

Methods:

1. Bacterial strains

E. coli isolates obtained from children with acute diarrhea who attended hospitals across Thailand during years 2008-2009 were included in this study. *E. coli* isolates were confirmed by standard methods [9].

2. Identification of Enteroaggregative *E. coli* by HeLa cells adherence assay

E. coli isolates were identified for EAEC by HeLa cell-adherence assay as described by Nataro *et al* [10]. HeLa cells were grown to 70-80% confluence in DMEM with 10 % fetal bovine serum and antibacterial-antimycotics (100 U of penicillin, 100 μg of streptomycin, and 0.25 μg/ml of amphotericin B in 0.85% saline) on circular cover slips of 13 mm diameter in 24-well tissue culture plates at 37°C in 5% CO_2. Cells were washed twice with 500 μl of phosphate-buffered saline (PBS), and 500 μl of fresh DMEM with 1% alpha-D-mannoside were added into each well. Ten microlitre of overnight-grown *E. coli* in LB-broth ($2x10^6$ cells) were inoculated into the wells. After 3 h incubation, the wells were washed thoroughly three times with PBS to remove non-adherent bacteria and air-dried completely. The cover slips in the wells were fixed with 100% methanol for 10 min, stained with 10% Giemsa stain for 30 min, and then air-dried completely. The cover slips were mounted on glass slides and examined under a light microscope. Each assay was done in duplicate. The adherence pattern was observed under light microscope. Three distinct adherence patterns were identified according to the descriptions of Nataro. Aggregative adherence pattern (AA) was characterized by the appearance of

stacked-brick clumps of bacteria on the surface of HeLa cells as well as on the cover slip. Localized adherence pattern (LA) was characterized by the formation of microcolonies on the cell surface but rarely on the cover slip. Diffuse adherence pattern (DA), the surface of cells were covered with individual bacteria. Non-adherence, only few bacteria adhered to the cells. *E. coli* isolates that adhered to HeLa cells in an AA pattern were identified as EAEC.

3. Determination of virulence-associated genes of EAEC by PCR.

Bacterial cell suspension (10^5 to 10^6 organisms per ml), which prepared by suspending colonies on overnight-grown LB agar plate in 100 μl of sterile distilled water, were boiled for 20 min and spin down to obtain template DNA in the supernatant. The PCR assay was carried out in 0.2 ml microcentrifuge tubes with 25 μl of reaction mixture consisting of PCR buffer, dNTPs, primers specific for genes encoding AAF/I (*agg*A), AAF/II (*aaf*A), AAF/III (*agg3*A), EAST1 (*ast*A) and Pet (*pet*), *Taq* DNA Polymerase, and template DNA. The reaction mixtures were run in a thermal cycler (model 9700, Perkin-Elmer Corp). The amplified DNA products were resolved by agarose gel electrophoresis at a constant voltage of 100 until the front dye had migrated approximately two-thirds of the length of the gel. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min, then rinsed twice with water for 10 min each. DNA fragments were visualized under UV light at 320 nm and photographed.

In addition, *E. coli* isolates were also ruled out of ETEC, EIEC, STEC, and EPEC by multiplex PCR as described by Fumiaki *el al* [11].

4. Determination of HeLa cells invasion assay

EAEC strains were tested for their ability to invade HeLa cells as described by Benjamin et al [20]. HeLa cells ($2x10^5$ cells) were grown to 70-80% confluence in DMEM with 10% fetal bovine serum and antibacterial-antimycotics (100U of penicillin, 100 μg of streptomycin, and 0.25 μg/ml of amphotericin B in 0.85% saline) in 24-well tissue culture plates (Nalge Nunc) at 37°C in 5%CO_2. HeLa cells in monolayer were washed twice with 500 μl of PBS, and 500 μl of fresh DMEM with alpha-D-mannoside at a final concentration of 1% was added into each well. At the same time, *E. coli* strains were cultured in 3 ml of LB broth at 37°C for 18 h. The well-grown bacterial culture were washed twice with PBS and reconstituted to a concentration of $1x10^8$ cells/ml. Twenty microlitre of bacterial suspension was inoculated into each tissue cultured well. After 2 h of incubation, the HeLa cells were washed three times

with PBS, and incubated for an additional 1 h in 500μl of fresh DMEM containing gentamicin (Difco) at the final concentration of 100 μg/ml. The infected cells were washed three times with PBS, lysed by adding 500 μl of sterile distilled water, and left at room temperature for 30 min. Serial dilutions of cell lysate were plated on LB agar for overnight incubation at 37°C to determine colony-forming unit (CFU), which is equivalent to the number of viable intracellular bacteria. The assays were done three times, with each time in duplicate. The percent invasion was calculated from the CFU of the original inoculum of bacteria and the mean CFU recovered from the HeLa cell lysates. The mean % invasion of three independent assays were normalized and scored as 0-10. EPEC strain E 1228 and JM 109 were used as positive and negative controls, respectively.

5. Antimicrobial susceptibility test

Antimicrobial susceptibility test of EAEC strains were tested with 12 antimicrobial disks including Amikacin (AK, 30 ug), Ampicillin (AMP, 10 ug), Amoxicillin/clavulanic acid (AMC, 30 ug), Cefotaxime (CTX, 30 ug), Ceftazidime (CAZ, 30 ug), Cefuroxime (CXM, 30 ug), Cephalothin (KF, 30 ug), Chloramphenicol (C, 30 ug), Sulphamethoxazole/Trimethoprim (SXT, 25 ug), Gentamicin (GN, 10 ug), Norfloxacin (NOR, 10 ug), and Tetracycline (TE, 30 ug) by agar disk diffusion method as described by National Clinical Laboratory Standard (2008). The suspected ESBL-producing EAEC strains were then confirmed with a combination antimicrobial disk of ceftazidime(30ug), ceftazidime/clavulinic acid(30/10ug) and cefotaxime(30ug), cefotaxime/clavulinic acid(30/10ug).

Results:

A total of 4,008 *E. coli* isolates obtained from children with diarrhea symptoms during the year 2008 and 2009 were examined in this study. EAEC strains were identified by HeLa cells adherence assay. EAEC strains were found in 268 of the 4008 (6.7%) *E. coli* isolates. The isolation rates of EAEC were about 7.6 % and 4.8 % in year 2008 and 2009.

EAEC strains were then determined for the presence of genes encoding AAF/I (*agg*A), AAF/II (*aaf*A), AAF/III (*agg3*A), EAEC heat stable enterotoxin (EAST1, *ast*A) and Plasmid encoded enterotoxin (Pet, *pet*) by a multiplex PCR. About 27%, 1%, and 3% of EAEC were *aggA*,

aafA, and *agg3A* positive, respectively; whereas, 68% of EAEC were AAF genes negative. Regarding the prevalence of enterotoxin genes, *astA* and *pet* genes were found in about 20%, and 12 % of EAEC, 9% of EAEC were both *astA* and *pet* genes positive as shown in table1.

EAEC strains were randomly selected and assayed with HeLa cells. The mean % invasion of the test strains were normalized and scored. Of 55 EAEC strains tested, 10.9% were found to be highly-invasive, 12.7% were invasive, and 76.4% were non-invasive. The CFU/well of highly-invasive EAEC and invasive-EAEC strains decreased gradually from 0 to 6 h of post-infection, with a marked decrease at 24 h of post-infection as shown in figure 1.

EAEC strains were tested for antimicrobial susceptibility by agar disk diffusion. About 90% of 192 EAEC strains were resistant to antimicrobial drug. EAEC strains were 90%, 76%, and 73% resistant to AMP, TE, and SXT, respectively. Details of antimicrobial resistant are shown in table2. About 72% of EAEC were multi drug (more than 3 different drugs) resistance. In addition, about 7% of EAEC strains were confirmed as ESBL-producing.

Discussion:

In this study, EAEC strains were investigated to determine the carriage of genes that encode for virulence-associated factors, such as *aggA* (AAF/I) and *aafA* (AAF/II), *agg3A* (AAF/III), *astA* (EAST1) and *pet* (Pet), by multiplex PCR. It was found that 32% were AAF genes positive. The majority of EAEC (~68%) were AAF genes negative. In addition, *astA* and *pet* were found in about 20% and 12% of EAEC strains. Taken together, the majority (47%) of EAEC from this study were both AAF and enterotoxin genes negative.

These results were somewhat different from other studies. Bouzari *et al.* [12] had shown by hybridization that among 98 EAEC strains from Iranian children, 8% were positive with AAF/I probe, 25% with AAF/II probe, and 5% with AAF/I and AAF/II probes. Ellis *et al.* [13] detected AAF/I and AAF/II in 19% and 8% of 115 EAEC strains associated with childhood diarrhea in Sao Paulo, Brazil. In contrast, Okeke *et al.* [14] detected AAF/I and AAF/II genes in 63% and 35% of 131 EAEC strains isolated from children in southwestern Nigeria. Czeczulin *et al.* [15] found that 32% of EAEC strains from various epidemiologic studies hybridized with AAF/I probe, and 18% hybridized with AAF/II probe. In addition,Vila *et al.* [16] showed that AAF/I and AAF/II were detected, respectively, in 0% and 8.7% of 23 diarrhea-associated EAEC strains from Spanish travelers to developing countries. Thus, the

prevalence of AAF in EAEC appears to vary substantially as it ranges from 0 to 47.2% for AAF/I and 8 to 35% for AAF/II. That ~68% of EAEC in this study were negative for *aggA*, *aafA*, and *agg3A*, is in agreement with those reports of Vila [16] and Ellis [13] that the majority of EAEC strains were negative to AAF/I and AAF/II probes. Therefore, these results strongly suggest the presence of alternative adherence factor(s) in the expression of AA phenotype.

The role of EAST1 (*astA*) and Pet (*pet*) in intestinal secretion and enterotoxicity has not yet been determined in humans. In this study, *astA* and *pet* were found in about 20% and 12% of EAEC strains. *astA* was found in 38% of EAEC strains associated with diarrhoea in Iranian children [12]. Savarino et al. [6] demonstrated that *astA* was found in approximately 40% of EAEC and in the similar proportion of non-pathogenic *E. coli* strains. Other *E. coli* categories, most notably EHEC, elaborate EAST1 with a higher frequency than EAEC [6]. Vila et al. [16] demonstrated that *astA* was detected in EAEC, diffuse adherent *E. coli*, and non diarrhoeagenic *E. coli* with more frequently in strains from the control group than from the case group. Thus, the potential of EAST1 to cause the disease is controversial. It is possibly that unless additional virulence factor(s) is present, EAST1 alone is insufficient to cause diarrhea.

In an outbreak of EAEC diarrhoea in Mexico [17], Pet was identified in sera from patients, suggesting its potential role in causing disease. Isolation rate of *pet* was variable geographically. Therefore, significant questions persist in understanding of EAEC epidemiology and pathogenesis. It has led some investigators to doubt the pathogenicity of EAEC.

Cravioto et al.[18] demonstrated that 33% of EAEC isolates were obtained from children with persistent bloody diarrhoea. In addition, 2 of the EAEC strains had invasive ability, although no further details were given. No other studies that have documented the association of EAEC strains with persistent diarrhoea have provided clinical descriptions of the diarrhoea to further assess the association of bloody diarrhoea with EAEC strains. HeLa cell-invasion assay in this study showed though EAEC strains are able to invade, or most probably internalized by the cells, they are unable to survive or multiply within the cells.

Nguyen et al [19] had studied the antimicrobial susceptibility of EAEC strains in Hanoi, he reported that 86.4% of Diarrheagenic *E. coli* strains were resistance to AMP, 88.3% were resistant to SXT, and multi drug resistance were found in 91.8% of EAEC strains. This present study also demonstrated that strains 90% and 73% of EAEC were resistant to AMP, and SXT,,

and more than 90% of EAEC in Thailand were multi drug resistance. These results indicated the spread of antimicrobial resistant strains of Diarrheagenic E. coli in this region. The genetic analysis of multi drug resistant and ESBL-producing needs to be further characterized.

References

1. Nataro JP, Steiner T, Guerrant RL. Enteroaggregative *Escherichia coli*. *Emerg Infect Dis*. 1998; 4: 251-61.

2. Itoh Y, Nagano I, Kunishima M, Ezaki T. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. *J Clin Microbiol*. 1997; 35: 2546-50.

3. Nataro JP, Deng Y, Maneval DR, German AL, Martin WC, Levine MM. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. *Infect Immun*. 1992; 60: 2297-304.

4. Czeczulin JR, Balepur S, Hicks S, et al. Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli*. *Infect Immun*. 1997; 65: 4135-45.

5. Christine Bernier, Pierre Gounon,[2] and Chantal Le Bouguénec. Identification of an Aggregative Adhesion Fimbria (AAF) Type III-Encoding Operon in Enteroaggregative *Escherichia coli* as a Sensitive Probe for Detecting the AAF-Encoding Operon Family. *Infect Immun*. 2002; 70: 4302-11.

6. Savarino SJ, Fasano A, Watson J, et al. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of E. coli heat-stable toxin. *Proc Natl Acad Sci U S A*. 1993; 90: 3093-7.

7. Eslava C, Navarro-Garcia F, Czeczulin JR, Henderson IR, Cravioto A, Nataro JP. Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli*. *Infect Immun*. 1998; 66: 3155-63.

8. Ratchtrachenchai, O., Subpasu, S., Hayashi, H., and Ba-Thein, W. 2004. Prevalence of

Childhood Diarrhoea-Associated *Escherichia coli* in Thailand. J Medical Microbiol, 53, 1–7.

9. Ewing WH. Edwards and Ewing's Identification of Enterobacteriaceae. *The Genus Escherichia*. 4 th ed. New York: Elsevier Science Publishing Co., Inc.; 1986: 93-134.

10. Nataro JP, J.B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M.M. Levine. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr. Infect. Dis. J.* 1987; 6: 829-831.

11. Fumiaki I, T. Ogino, K. Itoh, and H. Watanabe. Differentiation and detection of pathogenic determinants among diarrheagenic *Escherichia coli* by polymerase chain reaction using mixed primers. Nippon-rinshoh. 1992; 50: 343-347.

12. Bouzari S, Jafari A, Azizi A, Oloomi M, Nataro JP. Short report: characterization of enteroaggregative *Escherichia coli* isolates from Iranian children. *Am J Trop Med Hyg*. 2001; 65: 13-4.

13. Elias WP, Suzart S, Trabulsi LR, Nataro JP, Gomes TA. Distribution of *aggA* and *aafA* gene sequences among *Escherichia coli* isolates with genotypic or phenotypic characteristics, or both, of enteroaggregative *E. coli*. *J Med Microbiol*. 1999; 48: 597-9.

14. Okeke IN, Lamikanra A, Czeczulin J, Dubovsky F, Kaper JB, Nataro JP. Heterogeneous virulence of enteroaggregative *Escherichia coli* strains isolated from children in Southwest Nigeria. *J Infect Dis*. 2000; 181: 252-60.

15. Czeczulin JR, Whittam TS, Henderson IR, Navarro-Garcia F, Nataro JP. Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. *Infect Immun*. 1999; 67: 2692-9.

16. Vila J, Gene A, Vargas M, Gascon J, Latorre C, Jimenez de Anta MT. A case-control study of diarrhoea in children caused by *Escherichia coli* producing heat-stable enterotoxin (EAST-1). *J Med Microbiol*. 1998; 47: 889-91.

17. Eslava C, Navarro-Garcia F, Czeczulin JR, Henderson IR, Cravioto A, Nataro JP. Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli*. *Infect Immun*. 1998; 66: 3155-63.

18. Cravioto A, Tello A, Navarro A, et al. Association of *Escherichia coli* HEp-2 adherence

patterns with type and duration of diarrhoea. *Lancet*. 1991; 337: 262-4.

19. Trung Vu Nguyen, Phung Van Le, Chinh Huy Le, and Andrej Weintraub. Antibiotic Resistance in Diarrheagenic *Escherichia coli* and *Shigella* Strains Isolated from Children in Hanoi, Vietnam. Antimicrobial Agents and Chemotherapy, February 2005, p. 816-819, Vol. 49, No. 2.
20. Benjamin P, Federman M, Wanke CA. Characterization of an invasive phenotype associated with enteroaggregative *Escherichia coli*. *Infect Immun*. 1995; 63: 3417-21.

Table 1. Aggregative adherence fimbriae genes and enterotoxin genes of 268 EAEC strains as determined by PCR.

Aggregative fimbriae genes			Enterotoxin genes		No.(%)
*agg*A	*aaf*A	*agg3*A	*ast*A	*pet*	of strains
+	-	-	-	-	68 (25.4)
+	-	-	+	-	6 (2.2)
-	+	-	+	+	2 (0.7)
-	+	-	-	+	1 (0.4)
-	-	+	+	-	9 (3.4)
-	-	-	+	-	23 (8.6)
-	-	-	+	+	22 (8.2)
-	-	-	-	+	10 (3.7)
-	-	-	-	-	127 (47.4)
74 (27.6)	**3 (1.1)**	**9 (3.4)**	**62 (23.1)**	**35 (13.1)**	

Table 2. Antimicrobial resistant of EAEC strains isolated from children in year 2008-2009.

Type of antimicrobial	No. (%) of antimicrobial resistant EAEC strains		
	2008 (*n=112*)	**2009** (*n=61*)	**Total** (*n=173*)
AK	2(1.8)	0(0)	2(1.2)
AMP	100(89.3)	56(91.8)	156(90.2)
AMC	7(6.2)	4(6.6)	11(6.3)
CTX	7(6.2)	4(6.6)	11(6.3)
CAZ	5(4.5)	1(1.6)	6(3.5)
CXM	8(7.1)	5(8.2)	13(7.5)
KF	17(15.2)	14(22.9)	31(17.9)
C	49(43.7)	23(37.7)	72(41.6)
SXT	76(67.8)	51(83.6)	127(73.4)
CN	25(22.3)	13(21.3)	38(22.0)
NOR	1(0.9)	1(1.6)	2(1.1)
TE	88(78.6)	44(72.1)	132(76.3)

Note : Amikacin (AK, 30 ug), Ampicillin (AMP, 10 ug), Amoxicillin/clavulanic acid (AMC, 30 ug), Cefotaxime (CTX, 30 ug), Ceftazidime (CAZ, 30 ug), Cefuroxime (CXM, 30 ug), Cephalothin (KF, 30 ug), Chloramphenicol (C, 30 ug), Sulphamethoxazole/Trimethoprim (SXT, 25 ug), Gentamicin (GN, 10 ug), Norfloxacin (NOR, 10 ug), Tetracycline (TE, 30 ug)

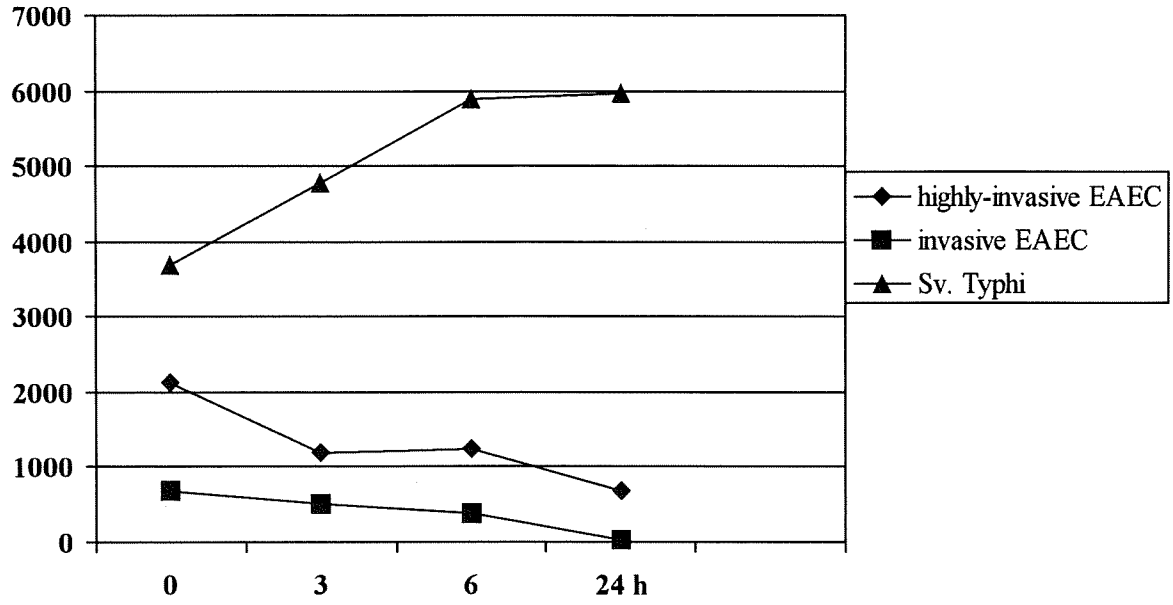

Figure 1. Intracellular survival in HeLa cells of highly-invasive and invasive EAEC, and *Salmonella* serovar Typhi.

Development and Evaluation of Multilocus Variable-Number Tandem Repeat Analysis for Fine Typing and Phylogenetic Analysis of *Salmonella enterica* Serovar Typhimurium

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Abstract

A total of 39 variable number of tandem repeat (VNTR) loci were explored in silico from five sequenced *Salmonella enterica* serovar Typhimurium strains. Sixteen of the VNTRs were polymorphic for the number of repeats in 40 diverse isolates tested. These 16 VNTRs were further tested on panels of 183 diverse isolates, 203 closely related isolates and 54 isolates from seven outbreaks, to assess their allelic diversity, variability and stability, to compare the discriminatory power of pulsed-field gel electrophoresis (PFGE) and multilocus VNTR analysis (MLVA) and to evaluate the usefulness of MLVA data in delineating phylogenetic structure among isolates. The evaluations revealed that five of the 16 VNTRs had diversity values greater than 0.5, and three (STTR5, STTR6 and STTR10) were hypervariable. The results obtained from the outbreak isolates suggested that the 16 VNTRs were considerably stable in isolates recovered during a normal outbreak time course. MLVA4, based on the four most variable VNTRs, exhibited a better resolving power over PFGE in discriminating among isolates, in particular among the closely-related isolates. MLVA5, which is based on five VNTRs and has been widely used in many European laboratories, displayed a level of discrimination close to MLVA4. The phylogenetic tree established using the MLVA16 profiles presented four distinct clusters, which were associated with four different phage types. Therefore, MLVA based on four or five highly variable VNTRs can be sufficient to supplement or replace PFGE for outbreak investigation and surveillance of *S.* Typhimurium infection, and MLVA data based on 16 VNTRs can be useful in establishing clonal structures among isolates.

Keywords: Salmonella enterica serovar Typhimurium; variable number tandem repeat (VNTR); multilocus VNTR analysis (MLVA); disease surveillance; phylogenetic analysis

1. Introduction

Salmonella enterica serovar Typhimurium is a broad–host range zoonotic pathogen, a major cause of human gastroenteritis, and transmitted primarily via contaminated food or, occasionally, via pets (Centers for Disease Control and Prevention, 2005) or from person to person (Alam et al., 2005). Diffused outbreaks or even multi-national outbreaks of *S.* Typhimurium infections have frequently been reported in recent years, accompanied by the application of discriminative molecular typing methods in the detection of disease cluster and epidemiological investigation (Bruun et al., 2009; Fuller et al., 2008; Van Duynhoven et al., 2009).

Among the discriminative molecular typing methods, pulsed-field gel electrophoresis (PFGE) is a universal subtyping tool for bacterial pathogens. Several PFGE protocols have been standardized and implemented in the laboratories of PulseNet International, an international molecular subtyping network for foodborne disease surveillance (Swaminathan et al., 2006). Although PFGE is highly discriminative for most bacterial pathogens, in certain circumstances it is not sufficient to resolve epidemiologically unrelated isolates of monomorphic pathogens (Liang et al., 2007; Noller et al., 2003). Moreover, PFGE data are less useful than sequence-based methods, for example, multilocus sequence typing (Maiden et al., 1998), in delineating phylogenetic structure among isolates.

Bacterial genomes contain thousands of tandem repeat (TR) loci; a small portion of the loci are polymorphic in terms of the number of repeats across strains. The polymorphic loci, referred to as variable-number tandem repeat (VNTR) loci, possess a wide range of mutability (Grant et al., 2008; Noller et al., 2006; Vogler et al., 2006; Vogler et al., 2007), making VNTRs useful for assessing genetic relatedness among