

ENTEROAGGREGATIVE *ESCHERICHIA COLI* INFECTIONS AMONG CHILDREN IN A TERTIARY HOSPITAL IN THE PHILIPPINES

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Abstract. Enteroaggregative *Escherichia coli* (EAEC) is recognized both in developed and developing countries as an enteric pathogen. In the Philippines, reports on epidemiology and detailed characterization of EAEC are lacking. Moreover, there is no standard method for the diagnosis of EAEC despite its significant impact on public health. This study determined prevalence of EAEC infection among children, aged 4 months to 12 years, in a tertiary hospital, Ospital ng Makati, Makati City, Philippines using phenotypic, ultrastructural and real-time PCR (targeting EAEC *aap*) methods. From 100 stool samples, 36 EAEC strains were isolated from 26 inpatients and 10 outpatients. Characteristic stacked brick-like aggregative adhesion of bacteria to HEp-2 cells and human cecal and ileal mucosa were evident in semi-thin and ultrastructural examinations. Clinical characteristics commonly associated with EAEC infection were mild to moderate dehydration, watery stool and persistent diarrhea. All EAEC strains were susceptible to amikacin, imipenem and piperacillin-tazobactam. This study highlights the usefulness of real-time PCR as an alternative to other PCR-based and HEp-2 adherence assays for rapid and specific identification of EAEC in a clinical setting.

Keywords: enteroaggregative *Escherichia coli*, *aap*, adherence assay, antibiogram, diarrhea, HEp-2 cell line, malnutrition, Philippines

INTRODUCTION

Despite much progress in understanding the pathogenesis and management of diarrhea-causing *Escherichia coli* strains since its first implication in the 1920's (Nataro and Kaper, 1998), its

prevalence in clinical infections remains high. The bacteria characteristically colonize gastrointestinal tract of infants within hours after birth and, although the infection is generally deemed harmless, certain host or strain conditions can enable pathogenesis (Nataro and Kaper, 1998).

The defining feature of enteroaggregative *E. coli* (EAEC) is its ability to elicit a characteristic stacked brick-like aggregative adherence to HEp-2 cells due to a fimbria, whose gene *aggR* is not

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present to other *E. coli* strains (Nataro *et al*, 1992). Among the diarrhea-causing *E. coli* strains, EAEC has been increasingly recognized recently in both developing and developed countries (Kahali *et al*, 2004; Regua-Mangia *et al*, 2009; Jin *et al*, 2013) as an important pathogen-causing diarrhea in children (Kahali *et al*, 2004; Jensen *et al*, 2014).

Epidemiological investigations involving EAEC conducted in the Philippines are still lacking. Based on the 2010 Annual Report – Field Health Service Information System of the Department of Health, the Philippines (National Epidemiology Center, 2010), acute watery diarrhea is the fifth leading cause of morbidity. However, the causative agents can range from bacteria, viruses to parasites, which have not been clearly defined in most tertiary clinical settings in the country. Risk factors for the development of EAEC infection are similar to those of other diarrheagenic *E. coli* (DEC) including improper food handling, food contamination and poor hygiene. Moreover, there is presently no consensus method for EAEC diagnosis even though it has a significant impact on public health (DuPont and Ericsson 1993; Nataro and Kaper, 1998; Flores and Okhuysen, 2009).

Hence, this study determined the prevalence of EAEC among children with and without diarrhea in a tertiary hospital, Ospital ng Makati (OSMAK), Philippines using phenotypic, molecular and ultrastructural characterization. In addition, the antibiotic resistance patterns of all *E. coli* isolates were determined.

MATERIALS AND METHODS

Study population and sample collection

One hundred stool samples were collected from 4 months to 12 years old

children, either inpatients ($n=50$) or outpatients ($n=50$). Inpatients were hospitalized due to acute diarrhea, characterized by the occurrence of three or more episodes of loose and watery stools within 24 hours and who had not taken any antimicrobial agent one week prior to the study. Outpatients were composed of controlled group at the pediatric outpatient department for reasons other than diarrhea and who showed no symptoms of any gastrointestinal infection for at least 30 days prior to the study. Stool samples were collected in standard sterile containers using standard hospital protocols. Sample collections were carried out from October to December 2013 at the Ospital ng Makati (OSMAK), Philippines.

The study protocol was approved by the Ethics Institutional Review Board of OSMAK (permit no. 2013-008). Prior written consents were sought from parents/legal guardians before the children were enrolled in the study.

Demographic data of subjects

Information of each subject, viz. age, gender, nutritional status using Waterlow classification (Rao and Kanade, 1988), and (for inpatients) stool consistency, duration of diarrhea, and other clinical symptoms such as vomiting, fever, and abdominal pain, were obtained upon enrollment. Data were analyzed using Pearson's chi-square test employing SPSS version 17 software (IBM, Armonk, NY). A p -value <0.05 is considered statistically significant.

Bacteria isolation and identification

Fecal swabs were streaked on MacConkey agar (MCA) (Becton, Dickinson, San Jose, CA) and incubated at 37°C for 24 hours. Only pink lactose-fermenting colonies (suggesting *E. coli*) were subjected to Vitek[®]2 test (BioMérieux, Marcy-l'Étoile,

France). Isolates with 99% probability match for *E. coli* in the GenBank database (www.ncbi.com) were used in subsequent experiments.

HEp-2 adherence assay

Adherence to HEp-2 cells (ATCC-CCC23) was performed as previously described with slight modifications (Cravioto *et al*, 1979). In brief, HEp-2 cells were grown overnight to 50% confluency on Dulbecco's minimum essential medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum, penicillin, streptomycin, and amphotericin in 8-well chamber plates at 37°C in 5% CO₂ atmosphere. Bacteria grown for 16 hours in trypticase soy broth (TSB) (Becton, Dickinson) without shaking were washed with phosphate-buffered saline (PBS) and 25µl aliquot of bacterial suspension was added to each chamber containing DMEM (Gibco, Thermo Fisher Scientific) supplemented with 1% mannose, incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The wells were washed with PBS, fixed with 70% methanol and stained with Giemsa. Each experiment was conducted in duplicate. *E. coli* (ATCC 25922) was used as positive control. Adherence was monitored using transmission electron microscopy (TEM). Bacteria treated HEp-2 cells were rinsed with PBS and fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, embedded in a low viscosity epoxy resin, and 1µm sections stained with methylene blue and examined under an inverted light microscope (400x magnification). Ultra-thin sections (700-800 nm) of selected areas were stained with uranyl acetate and lead citrate and examined by TEM, (JEOL JEM-F200 F2, Tokyo, Japan).

Intestinal mucosa adhesion assay

The intestinal mucosa adhesion assay was performed as described by Browning

and Trier (1969) and modified by Knutton *et al* (1987) with slight modifications. In short, normal ileal and cecal mucosal biopsies obtained with prior written informed consent from a female adult undergoing routine colonoscopy were placed on sterile foam sponge immersed in bicarbonate-buffered culture medium (NCTC-135- DMEM; Gibco, Thermo Fisher Scientific) containing 10% calf serum) adjusted to thinly cover the villous surface of the tissues. A 25µl aliquot of bacterial broth culture was placed on the mucosal surface of the biopsy sample and incubated for 12 hours at 37°C in 5% CO₂ atmosphere. Biopsy samples were washed with fresh medium, fixed in 10% formalin and 2.5% glutaraldehyde and underwent routine histopathologic and ultrastructural examinations.

Detection of EAEC *aap*

Total DNA was extracted from pure bacterial isolates (including positive control EAEC 042 strain and negative control *E. coli* K12) using QIAamp DNA Extraction Kit (Qiagen, Hilden, Germany). PCR mixture (25µl) contained 0.25 µmol/l primers AapF (5'-CTT GGG TAT CAG CCT GAA Tg-3') and AapR (5'-AAC CCA TTC GGT TAG AGC AC-3') (Roche *et al*, 2010), 20µl of BioRadiQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and 5µl of DNA (replaced with distilled water in negative control). Thermocycling was performed in a Bio-Rad® CFX-96 real-time thermal cycler (Bio-Rad) as follows: 95°C for 5 minutes; followed by 40 cycles of 95°C for 20 soconds, 55°C for 20 seconds and 72°C for 20 seconds. Negative result is considered if no C_T (threshold cycle) was obtained after 40 cycles. A melting curve analysis (T_m = 65°C) was conducted on all positive samples to confirm specific production of the expected 232-bp amplicon.

Table 1
Demographic data and prevalence of EAEC in the study population at Ospital ng Makati, Philippines.

Characteristic	Inpatient		Outpatient		Total	
	Total (n = 50)	EAEC- positive (%) (n = 26)	Total (n = 50)	EAEC- positive (%) (n = 10)	Total (%) (n = 100)	EAEC- positive (%) (n = 36)
Age group (years)						
<1	5	1 (4)	5	0 (0)	10 (10)	1 (3)
1-6	36	22 (85)	37	8 (80)	73 (73)	30 (83)
7-12	9	3 (11)	8	2 (20)	17 (17)	5 (14)
Sex						
Male	20	9 (35)	17	0 (0)	37 (37)	9 (25)
Female	30	17 (65)	33	10 (100)	61 (63)	27 (75)

Antimicrobial susceptibility testing

Antibiotic susceptibility testing (AST) of all isolates was performed using a minimum inhibitory concentration (MIC) microdilution method in an automated Vitek[®]2 instrument (BioMérieux). The Vitek[®]2 AST-GN67-413399 cards contained the following antibiotics: amikacin (30 µg), ampicillin (10 µg), ampicillin-sulbactam (10 µg), cefazolin (30 µg), cefepime (30 µg), ceftriaxone (30 µg), cefuroxime-sodium (30 µg), cefuroxime-axetil (30 µg), levofloxacin (5 µg), gentamicin (10 µg), nitrofurantoin (300 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tobramycin (10 µg), piperacillin-tazobactam (100/10 µg), ciprofloxacin (5 µg), and imipenem (10 µg). The MICs for each antibiotic and extended spectrum β-lactamase (ESBL) production were evaluated according to the Clinical and Laboratory Standard Institute criteria (CLSI, 2013). *E. coli* ATCC[®] 25922 was included as a negative control.

RESULTS

Prevalence of EAEC in the study population

Seventy-three percent of the study

group were between one to six years old and 63% females (Table 1). Among the 100 subjects, 36 (83% between 1-6 years of age) were positive for both EAEC *aap* (Table 1) and HEp-2 adherence (Fig 1). Semi-thin sections of representative HEp-2 adhesion-positive samples showed the characteristic stacked brick-like pattern both on the surface and between HEp-2 cells (Fig 2). On the other hand, ultrastructural observations demonstrated aggregates of rod-shaped bacteria (2.0-2.5 µm long and 0.5-0.8 µm in diameter) with distinct nucleoid, cell wall, glycocalyx and aggregative adherence fimbriae (AAF), while the negative control *E. coli* ATCC 25922 exhibited non-aggregative characteristics (Fig 3).

There is no significant difference between the genders of positives. Among children positive for EAEC, 26 inpatients (72%) experienced mild (46%) to moderate (31%) malnutrition; and mild (38%) to moderate (38%) dehydration (Table 2). On the other hand, all EAEC-positive outpatients (n=10) significantly lacked indicators for malnutrition ($p=0.001$) and dehydration ($p=0.001$). Watery stool was

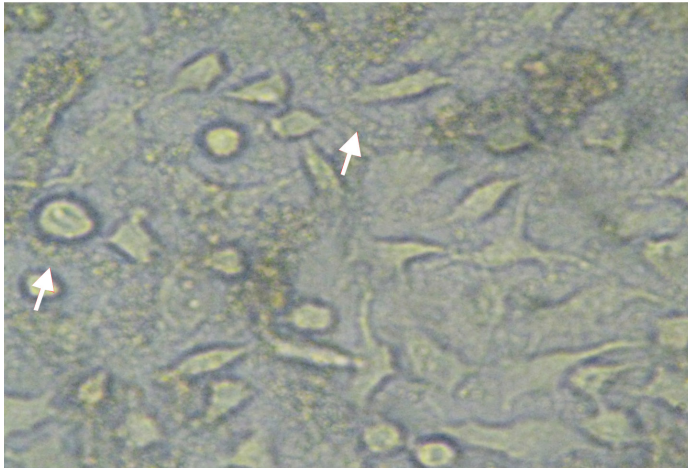


Fig 1—Micrograph of representative clinical EAEC adherence to HEp-2 cells. Arrows indicate characteristic stacked brick-like aggregative adhesion on surface and between HEp-2 cells. (Wet mount under inverted microscope, 400x magnification).

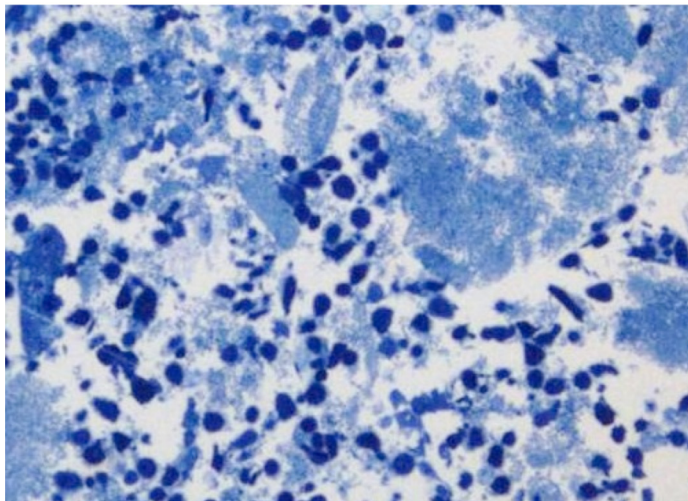


Fig 2—Micrograph of semi-thin section of representative clinical EAEC adherence to HEp-2 cells. (Methylene blue staining, 400x magnification).

found in 12/26 (46.15%) inpatients, followed by loose stool (38%) (Table 3). In addition, abdominal pain was noted to be the most common (65%) symptom, occurring alone (12%) or accompanied with

vomiting (59%) or fever (35%).

Intestinal adhesion studies

Light microscopy revealed aggregating rod-shaped bacteria on the surface of columnar epithelium and mucus material (Fig 4). Likewise, semi-thin sections showed mucus material with aggregates of rod-shaped bacteria (Fig 5A). Electron micrograph revealed aggregates of rod-shaped bacteria (1-2 μ m in length) supported by mucus material and associated with intact brush border, devoid of vesiculation (Fig 5B).

EAEC antibiogram

The antibiogram of EAEC clinical isolates revealed that three antibiotics, namely, amikacin, imipenem and piperacillin-tazobactam, were the most effective drug (100% susceptibility) (Table 4). The EAEC isolates were least susceptible to ampicillin and ampicillin/sulbactam, and less susceptible to some degree to the rest of the 15 tested antibiotics. Two EAEC isolates were capable of producing ESBL.

DISCUSSION

The results of this study establish the prevalence of EAEC with regard to acute pediatric diarrhea and its association with malnutrition in children in the Philippines. Other studies on the prevalence of EAEC infection among children revealed the proportion of children with diarrhea and infected with EAEC

Table 2
EAEC in stool samples of the study population at Ospital ng Makati, Philippines.

Characteristic	Inpatient (%) (n = 26)	Outpatient (%) (n = 10)	χ^2	p-value ^a
Malnutrition status			21.76	0.001
Normal	4 (15)	10 (100)		
Mild	12 (46)	0 (0)		
Moderate	8 (31)	0 (0)		
Severe	2 (8)	0 (0)		
Dehydration status			19.38	0.001
Normal	5 (19)	10 (100)		
Mild	10 (38)	0 (0)		
Moderate	10 (38)	0 (0)		
Severe	1 (4)	0 (0)		

^aSignificant at $p < 0.05$.

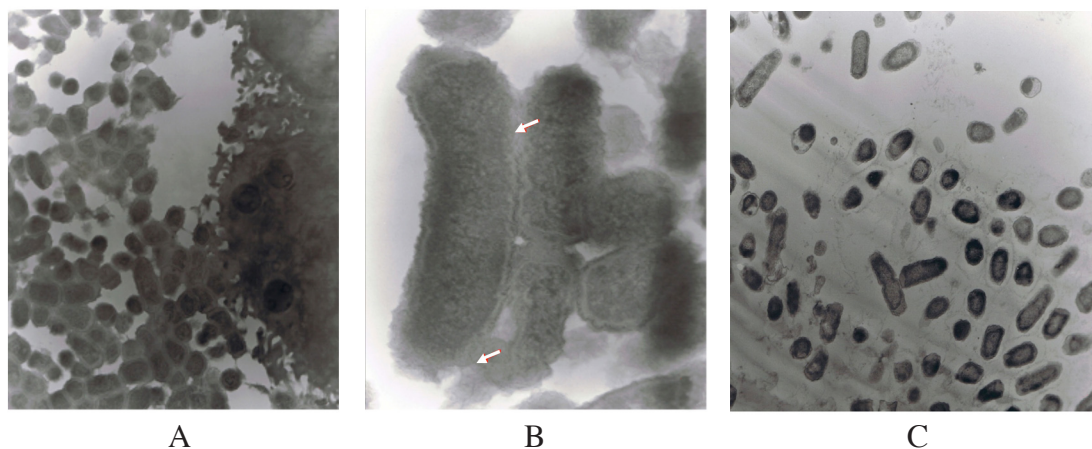


Fig 3—Transmission electron micrographs of representative clinical EAEC adherence to HEp-2 cells. A. 28,000x magnification. B. 105,000x magnification. Arrows indicate aggregative adherence fimbriae. C. Negative control (*E.coli* ATCC 25922) (14,000x magnification).

is unexpectedly higher (0.5-2 folds) than those without infection (Gonzales *et al*, 1997; Okeke *et al*, 2000; Pabst *et al*, 2003). The present study conducted at a tertiary hospital in the Philippines shows the prevalence of EAEC infection was relatively high (36%), the majority of which were isolated from inpatients. Association with malnutrition may have profound effects on intestinal absorption, which

might eventually lead to aggravation of the effects if not treated promptly. It is only appropriate that nutritional therapy should also be included in the regular medical treatment protocol.

While EAEC has been implicated in diarrheal diseases both in developing and developed countries, the burden of the problem is borne by the developing countries, where EAEC infection is cor-

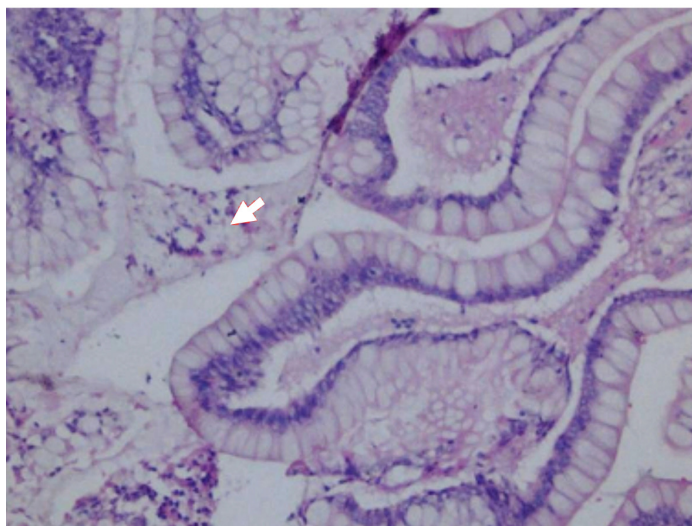


Fig 4—Micrograph of representative clinical EAEC adherence to human ileal mucosa. Arrow indicates aggregating rod-shaped bacteria on the surface of columnar epithelium and mucus material. (H & E staining, 400x magnification).

related with pediatric diarrheal illnesses and linked with malnutrition (Opintan *et al*, 2010; Estrada-Garcia and Navarro-Garcia, 2012; Kotloff *et al*, 2013). Roche *et al* (2010) emphasized the serious sequelae of persistent diarrhea for which EAEC is reportedly a predominant cause of malnutrition and compromised immunity, both of which predispose people to long-term disability or death from other reported causes. Hence, additional understanding of EAEC, its pathogenic mechanisms in various clinical scenarios and cost-effective interventions are urgently needed.

EAEC infection, with or without overt diarrhea, has profound effects on intestinal absorption, nutrition and childhood development as well as on global mortality (Huang *et al*, 2006; Opintan *et al*, 2010). Oral rehydration therapy has reduced the number of deaths from dehydration caused by infection with enteric pathogens, but it has not changed the morbidity

caused by such infection (Petri *et al*, 2008). Children with EAEC infection may develop acute watery diarrhea with or without passage of blood and mucus, abdominal pain, nausea, vomiting and low-grade fever (Huang and Dupont, 2004). Our study may imply that in local settings, the common clinical characteristics of EAEC infection involve mild to moderate dehydration, loose to watery stool, abdominal pain, and diarrhea of greater than two days in duration.

Prevalence and significance of EAEC infections may also depend on age (Pabst *et al*, 2003). Although various studies state other-

wise (Nataro *et al*, 2006; Chattaway *et al*, 2013), this variation may be due to strain diversity, differences in geographical locations, or by the presence of asymptomatic carriers (Pabst *et al*, 2003). In relation to its diagnosis, HEp-2 adherence assay remains the gold standard for identification of EAEC but is performed in only a few laboratories around the world because it requires special expertise and facilities. For this reason, Cerna *et al* (2003) developed a sensitive multiplex PCR specifically targeting AA plasmid, which, together with *aap* and *aggR* is present in most EAEC strains isolated from patients with diarrhea. In this study, we decided to identify EAEC via real-time PCR with *aap* as the target gene, since it is most frequently detected (Cerna *et al*, 2003; Bouzari *et al*, 2005; Regua-Mangia *et al*, 2009; Lima *et al*, 2012; Sumbana *et al*, 2015). However, since this dispersin-encoding gene can also be found in diffusely adherent *E. coli*

Table 3
Clinical characteristics of EAEC-infected inpatients at Ospital ng Makati, Philippines.

Characteristic	Inpatients (%) (n = 26)
Stool consistency	
Watery	12 (46)
Loose	10 (38)
Mucoid	3 (12)
Blood-tinged	1 (4)
Duration of diarrhea before admission	
<24 hours	4 (15)
24 to 48 hours	5 (20)
>48 hours	17 (65)
Clinical symptoms	
Abdominal pain only	3 (12)
Vomiting only	5 (19)
Fever only	4 (15)
Abdominal pain with vomiting	8 (31)
Abdominal pain with fever	4 (15)
Abdominal pain with fever and vomiting	2 (8)

Table 4
Antibiotic susceptibility of 36 EAEC isolates from the study population at Ospital ng Makati, Philippines.

Antibiotic	Number of isolates (%)		
	Susceptible	Intermediate	Resistant
Amikacin	36 (100)	0 (0)	0 (0)
Ampicillin	15 (42)	1 (3)	20 (55)
Ampicillin/Sulbactam	18 (50)	13 (36)	5 (14)
Cefazolin	31 (86)	0 (0)	5 (14)
Cefepime	33 (92)	2 (5)	1 (3)
Ceftriaxone	34 (94)	0 (0)	2 (6)
Cefuroxime-sodium	33 (91)	1 (3)	2 (6)
Cefuroxime-axetil	31 (86)	3 (8)	2 (6)
Ciprofloxacin	35 (97)	0	1 (3)
Gentamicin	33 (91)	1 (3)	2 (6)
Imipenem	36 (100)	0 (0)	0 (0)
Levofloxacin	34 (94)	1 (3)	1 (3)
Nitrofurantoin	33 (91)	1 (3)	2 (6)
Piperacillin/Tazobactam	36 (100)	0 (0)	0 (0)
Tobramycin	34 (94)	2 (6)	0 (0)
Trimethoprim-Sulfamethoxazole	22 (61)	0 (0)	14 (39)
ESBL	2 (6)		

ESBL, extended spectrum beta-lactamase.

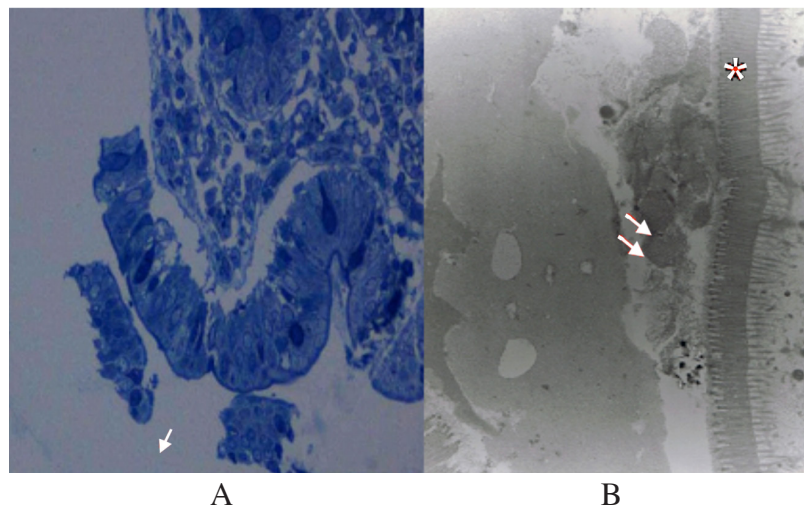


Fig 5—Micrograph (Methylene blue staining, 400x magnification) (A) and transmission electron micrograph (35,000x magnification) (B) of representative clinical EAEC adherence to human cecal mucosa. A. Arrow indicates mucus material with aggregates of rod-shaped bacteria. B. Arrows indicate aggregates of rod-shaped bacteria (1-2 μ m in length) supported by mucus material and associated with intact brush border, devoid of vesiculation. *Columnar epithelium with intact brush border membrane.

and a number of non-pathogenic *E. coli* (Monteiro *et al*, 2009), the adherence assay and ultrastructural studies performed supported the PCR results. This should simulate similar studies be conducted on other infections in clinical settings.

Most of the confirmed EAEC strains were isolated from children with diarrhea, and ten from the control group. This finding further strengthens previous reports that EAEC is not just seen among children with overt and explosive diarrhea but also among asymptomatic children (Steiner *et al*, 2000; Regua-Mangia *et al*, 2009). The heterogeneous nature of EAEC strains with regard to the organism-borne genetic factors with varying pathogenic effects may explain its inconsistent association with diarrheal diseases. Our study was not able to compare different virulence

genes of the isolates and their association with the severity of diarrhea because we were focused on a single gene target with histological examinations to detect EAEC.

The clinical strains in this study exhibited susceptibility to amikacin, imipenem and piperacillin-tazobactam, consistent with *in vitro* antimicrobial susceptibility of intra-abdominal infections (Chang *et al*, 2017) and even blood isolates of *E. coli* (Sutherland *et al*, 2016), where amikacin possesses high *in vitro* activity. Although antibiotic

therapy is not usually recommended in diarrheic patients, EAEC-infected patients may be given these treatments to preclude the progression of malnutrition (Trehan *et al*, 2013; Isanaka *et al*, 2016). A number of strains showed non-susceptibility to other known antibiotics tested, implying that a thorough and well-designed tandem antibiotic therapy be recommended to address the co-morbidity and preservation of the intestinal microflora of the patients. This study supports a customized drug therapy, especially to diarrheic patients, whenever necessary. Since this study is limited to a small cohort in one geographic area, we recommend that similar studies involving larger number of patients at different geographic areas be conducted to demonstrate the heterogeneity of EAEC strains and to determine their impact on

the mode of treatment.

In summary, this study revealed 36% diarrheic patients, aged <1-12 years, at Ospital ng Makati had EAEC infection. EAEC-affected children with malnutrition at a higher rate. Antimicrobial resistance profiling revealed the isolates were susceptible to amikacin, imipenem and piperacillin-tazobactam. These results should instigate other health providers to consider this pathogen in the treatment of acute and persistent diarrhea in children.

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