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Full Length Research Paper

Biochemical and molecular identification of enteroaggregative *Escherichia coli* associated with childhood diarrhea and antimicrobial susceptibility profile of the isolates in Egypt

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Molecular identification and antimicrobial susceptibility of enteroaggregative *Escherichia coli* (EAEC) associated with childhood diarrhea was done out in Egypt. The usefulness of quantitative biofilm assay in detection of EAEC was compared with multiplex polymerase chain reaction (PCR). One hundred and fifty cases of childhood diarrhea were divided into three groups; 50 cases of acute diarrhea (group I), 50 cases of persistent diarrhea (group II) and 50 cases of healthy subjects of matched age and sex as a control group (group III). *E. coli* was isolated and identified by conventional microbiological methods. EAEC was detected by multiplex PCR and quantitative biofilm assay. Antimicrobial susceptibility profile of the isolated EAEC strains was done using disc diffusion method. *E. coli* was isolated from 78% (39/50) cases of acute diarrhea and 76% (38/50) cases of chronic diarrhea. The results show no significant difference between the results of multiplex PCR and quantitative biofilm assay; in 77 *E. coli* isolates, 15 (19.5 %) generated positive results for EAEC with multiplex PCR for two the specific genes AggR and EAST and 12 (15.6 %) strains showed positive results for EAEC by quantitative biofilm assay. As regard the antimicrobial susceptibility profile of the isolated EAEC strains, the results show that 85.7 and 87.5% of the EAEC strains isolated from cases of acute and persistent diarrhea, respectively were sensitive to amikacin, 47.1 and 62.5% were sensitive to cefoperazone, 28.5 and 50.00% were sensitive to ceftriaxone and 42.8% and 62.5% were sensitive to imepenem, 28.5 and 12.5 % of the EAEC strains isolated from cases of acute and persistent diarrhea respectively were sensitive to Amoxicillin-Clavulanic. All the isolated EAEC strains (100.00%) were resistant to sulphamethoxole/trimethoprim. High incidence of EAEC associated diarrhea among pediatric cases in Egypt must be considered before decision of antimicrobial therapy. Quantitative biofilm assay can be simple, rapid and convenient method for detection of EAEC in comparison with molecular methods and can therefore be recommended as a rapid screening test for EAEC in clinical laboratories.

Key words: Enteroaggregative *Escherichia coli* (EAEC), infantile diarrhea, multiplex polymerase chain reaction (PCR), quantitative biofilm.

INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) have emerged as an important pathogen associated with endemic and epidemic diarrheal diseases in both industrialized and developing countries (Albert et al., 1999). In Egypt, about 16% of population is children under 5 years of age. Each child suffers, on the average, 3 bouts of acute diarrhea yearly, that is, 10 million children suffer 30 million episodes of acute diarrhea every year. Diarrhea accounts for 20-25% of deaths among children younger than five years. Diarrhea is a leading cause of under nutrition and poor growth, causing prolonged morbidity that may end fatally (El-Mougi 1999). EAEC strains are defined by their characteristic "stacked brick" aggregative adherence (AA) pattern to cultured epithelial cells (Nataro et al., 1992) and this is the basis of the assay considering the gold standard for EAEC identification. However, this technique requires specialized facilities and can therefore be performed only in reference laboratories. As alternative to this technique, a variety of phenotypic and molecular assays have been proposed (Wakimoto et al., 2004). Recently, a multiplex PCR assay for EAEC detection has been developed; one of these assays detects simultaneously three EAEC plasmid-borne genes: *aggR*, which encodes a central regulator involved in the expression of several EAEC virulence genes *(*Pass et al., *2000); aap*, which encodes the antiaggregation protein dispersin (Kimata et al., 2005) and *aatA*, which is part of a gene cluster that codes for a specific ATP-binding cassette transporter system (Sarantuya et al., 2004). These molecular techniques are of high costs and difficult to apply in clinical laboratories (Wakimoto et al., 2004; Sarantuya et al., 2004).

Thus, it is difficult to screen for EAEC among *E. coli* isolates from patients with diarrhea in clinical laboratories. The use of biofilm assays may be useful in overcoming these difficulties. Nataro and Kaper (1987) reported that EAEC produces a bacterial film on a polystyrene surface that could be easily visualized with Giemsa, a character which is used as a base for quantitative biofilm assay (Nataro and Kaper, 1987). The aim of this study was to evaluate the usefulness of the quantitative biofilm assay to screen the prevalence of EAEC among the clinical isolates causing acute and persistent diarrhea in pediatric cases and study the antimicrobial susceptibility profile of the isolated EAEC strains.

MATERIALS AND METHODS

After Research Ethical Committee approval and a written informed consent from parents of all participants in this research, this prospective randomized control study was conducted between 1/3/2012 to 1/3/2013 at Diarrhea and Malnutrition Unit in Pediatrics Department, Tanta University Hospital. The study was carried out on 150 cases divided into three groups: group I: 50 patients with persistent infantile diarrhea, group II: 50 patients with acute infantile diarrhea, group III: 50 healthy subjects of the same age group as a control group. Inclusion criteria: All infants suffering from acute or persistent diarrhea; Exclusion criteria: Antibiotic treatment for at least five days before this study, chronic disease and systemic infection.

Microbiological study (stool culture for isolation of *E. coli*)

Stool specimen were sent to microbiological laboratory as soon as possible for bacteriological study that include Gram stain smears to detect *E. coli* in stool specimens as Gram-negative bacilli, culture in aerobic facultative anaerobic incubator, in 37°C, for 24 - 48 h, on MacConkey's medium, and then the colonies were identified by biochemical reactions which include action on sugar media including lactose, sucrose, glucose, maltose, mannitol; action on triple sugar media and IMViC formula including (indole test, methyl red test, Voges proskaur test, citrate utilization test).

Multiplex PCR

The isolated strains of *E. coli* were also characterized by a multiplex PCR with below mentioned primers for the detection of two specific genes *aggR* (630 bp) and *east* (97 bp). The primers were chosen from a reference protocol (Kahali et al., 2004). For standardization purpose we used positive 042 strain and 044 strains as control strains.

Bacterial lysates were prepared by re-suspending a single colony in 1 ml of deionized water in a sterile 5 ml glass tube followed by boiling for 10 min at 95°C. After boiling the suspension is centrifuged at 10,000 rpm for 10 min and the supernatant solution is directly used as a template for PCR.

Each PCR tube contained 50 µl of reaction mix [(10x PCR buffer with $MgCl_2$; dNTP mix 2.5 mM each; 4 primers 10 mM each, which comprised of *aggR* 5' CTGGCGAAAGACTGTATCAT' 3 + 5' CAATGTATAGAAATCCGCTGTT' 3 and for *east* 5' CACAGTATATCCGAAGGC' 3 + 5' CGAGTGACGGCTTTGTAG' 3, Template lysate, sterile water, Taq polymerase (5U/l)] and total volume made up to 50 µl.

The solutions were then subjected to the following cycling conditions- denaturation 94°C/1 min, annealing 55 °C/1 min, extension 72°C/1 min, final extension 72° C/7 min in a thermal cycler. Then 10 µl of the PCR mixture was visualized by ethidium bromide staining after electrophoresis in 2% agarose gel in tris acetate -EDTA buffer.

Quantitative biofilm assay

To assess biofilm formation, we inoculated 200 µL of Dulbecco's modified Eagle's medium containing 0.45% glucose in 96-well flat-bottom microtiter polystyrene plates (Becton Dickinson, Franklin

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Table 1. Demographic and clinical characteristics of the studied groups in relation to EAEC infection.

Demographic and clinical characteristics			Quantitative biofilm assay			Chi-square	
			Negative to EAEC	Positive to EAEC	Total	X^2	P-value
Age	<2years	N (%)	60 (70.6)	12 (80.0)	72		
	>2years	N (%)	25 (29.4)	3 (20.0)	28	0.447	0.896
sex	Female	N (%)	41 (48.2)	7 (46.7)	48		
	Male	N (%)	44 (85.0)	8 (53.3)	52	0.523	0.774
Resident	Urban	N (%)	21 (24.7)	3 (20.0)	24		
	Rural	N (%)	64 (75.3)	12 (80.0)	76	1.336	0.241
Season	Summer and autumn	N (%)	60 (70.6)	9 (60.0)	69	1.447	0.335
	Winter and spring	N (%)	25 (29.4)	6 (40.0)	31		
Feeding pattern	Breast feeding	N (%)	53 (62.4)	4 (26.7)	57	12.336	0.001*
	Non breast feeding	N (%)	32 (37.7)	11 (73.3)	43		
Vomiting	Negative	N (%)	29 (34.1)	5 (33.3)	34	0.420	0.361
	Positive	N (%)	56 (65.9)	10 (66.7)	66		
Dehydration	No dehydration	N (%)	45 (52.9)	6 (40.0)	51	1.632	0.147
	Some dehydration	N (%)	34 (40.0)	9 (60.0)	43	1.985	0.256
	Severe dehydration	N (%)	6 (7.1)	0 (0.0)	6	1.996	0.062
Fever	Low	N (%)	50 (58.8)	9 (60.0)	59	1.669	0.255
	High	N (%)	35 (41.2)	6 (40.0)	41		
Mucus	Negative	N (%)	58 (68.2)	1 (6.7)	59	14.668	0.001*
	Positive	N (%)	27 (31.8)	14 (93.3)	41		
RBCs	Negative	N (%)	66 (77.7)	4 (26.7)	70	15.575	0.001*
	Positive	N (%)	19 (22.4)	11 (73.3)	30		
Pus	Negative	N (%)	33 (38.8)	7 (46.7)	40	1.574	0.225
	Positive	N (%)	52 (85.0)	8 (15.0)	60		

*Significant at P-value < 0.05.

Lakes, NJ) with 5 µL of an overnight Luria broth culture grown at 37°C with shaking. The sample was incubated overnight (18 hours) at 37°C and visualized by staining with 0.5% crystal violet for five minutes after washing with water. The biofilm was quantified after adding 200 µL of 95% ethanol, by an enzyme-linked immuno-sorbent assay plate reader at 570 nm. Strain EAEC 042 was used as a positive control and *E. coli* HB101 was used as a negative control. All EAEC strains showed absorbance >0.2 (Sarantuya et al., 2004).

Antibiotic sensitivity test

Antibiotic susceptibility testing of EAEC isolates was performed using the standardized disc agar diffusion method (Oxoid-England) using discs of Cefebime (30 µg), Amikacin (30 µg), Co-trimoxazol (25 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Amoxillin-clavulanic (10 µg) and Cefotriaxone, Cefoperazone (10µg). Interpretation of the results was done according to CLSI guidelines 2008.

Statistics

Statistical presentation and analysis of the present study was conducted, using Chi-square test by SPSS V.16.

RESULTS

The present work was carried out on fifty children suffering from acute diarrhea, their age ranged between 2 months and 6 years (mean ± SD: 2 ± 3.54), they were 27 males and 23 females and another fifty children suffering from persistent diarrhea were used, their age ranged between 2 months and 4 years (mean ± SD: 2 ± 5.14), they were 30 males and 20 females. All cases were attending Diarrhea and Malnutrition Unit in Pediatrics Department, Tanta University Hospital. Fifty normal healthy children of matched age and sex served as a control group. Demographic and clinical characteristics of the studied groups in relation to EAEC infection are presented in Table 1.

The results of this study show that *E. coli* represent the causative organism of infantile diarrhea in 39 out of 50 cases of acute infantile diarrhea (39%) and 38 out of 50 cases of persistent infantile diarrhea (38 %). None of the 50 control samples collected showed positive results with biofilm nor generated positive PCR for two specific genes tested.

The results showed that out of the total 77 *E. coli*

Table 2. Comparison between the result of quantitative biofilm assay test and multiplex PCR with regards to EAEC infection.

	Positive		Negative		Total		Chi-Square	
	N	%	N	%	N	%	X^2	P-value
Quantitative biofilm assay	12	12	88	88	100	100		
Multiplex PCR	15	15	85	85	100	100	0.168	0.682

Table 3. Antimicrobial susceptibility pattern of EAEC isolates from cases of acute diarrhea.

Antimicrobial agent	Resistant N (%)	Highly sensitive N (%)	Moderately sensitive N (%)
Amikacin	0 (0.0)	6 (85.7)	1 (14.2)
Amoxicillin-Clavulanic	5 (71.4)	0 (0.0)	2 (28.5)
Cefoperazone	(0.0)	4 (47.1)	3 (42.8)
Cefotrioxone	2 (28.5)	2 (28.5)	3 (42.8)
Ciprofloxacin	4 (47.1)	2 (28.5)	1 (14.2)
Cefibim	4 (47.1)	0 (0.0)	3 (42.8)
Sulphamethole/Trimethoprim	7 (100.0)	0 (0.0)	0 (0.0)
Imepenem	2 (28.5)	3 (42.8)	2 (28.5)

Table 4. Antimicrobial susceptibility pattern of EAEC isolates from cases with persistent infantile diarrhea.

Antimicrobial agent	Resistant N (%)	Highly sensitive N (%)	Moderately sensitive N (%)
Amikacin	0 (0.0)	7 (87.5)	1 (12.5)
Amoxicillin-Clavulanic	7 (87.5)	0 (0.0)	1 (12.5)
Cefoperazone	0 (0.0)	5 (62.5)	3 (37.5)
Cefotrioxone	2 (25.0)	4 (50.0)	2 (25.0)
Ciprofloxacin	5 (62.5)	3 (37.5)	0 (0.0)
Cefibim	7 (87.5)	0 (0.0)	1 (12.5)
Sulphamethole/Trimethoprim	8 (100.0)	0 (0.0)	(0.0)
Imepenem	3 (37.5)	(0.0)	5 (62.5)

isolates, 15 generated positive results with multiplex PCR for two specific genes *aggR* and *east*. By quantitative biofilm assay, 12 (80 %) strains showed positive results by Quantitative microtitre plate assay (P-value 0.682) (Table 2).

As regard the antimicrobial susceptibility profile of the isolated EAEC strains the results showed that 85.7 and 87.5% of the EAEC strains isolated from cases of acute and persistent diarrhea respectively were sensitive to Amikacin, 47.1 and 62.5% were sensitive to cefoperazone, 28.5 and 50.00% were sensitive to Ceftriaxone and 42.8 and 62.5% were sensitive to Imepenem. 28.5 and 12.5% of the EAEC strains isolated from cases of acute and persistent diarrhea respectively were sensitive to Amoxicillin-Clavulanic. All the isolated EAEC strains (100.00%) were resistant to Sulphamethoxole/Trimethoprim (Tables 3 and 4).

DISCUSSION

The importance of EAEC strains in public health around the world is becoming increasingly clear. The EAEC strains have been associated classically with persistent diarrhea (≥ 14 days) and with growth retardation in infants (Iwanaga et al., 2002). EAEC diarrhea involves bacterial aggregation, adherence to intestinal epithelial cells and elaboration of several toxigenic bacterial mediators. EAEC is primarily recognized as a cause of endemic and persistent childhood diarrhea in developing countries (Gascon et al.,

2000; Albert et al., 1999). Therefore, the detection of EAEC strains can make a significant contribution to public health in many areas. The present work was carried out on fifty children suffering from acute diarrhea their age ranged between two months and six years, they were 27 males and 23 females and fifty children suffering from persistent diarrhea their age ranged between two months to four years, they were 30 males and 20 females. All cases were admitted to Diarrhea and Malnutrition Unit in Pediatrics Department, Tanta University Hospital. Fifty normal healthy children of matched age and sex served as the control group. The results of this study showed that *E. coli* represent the causative organism of diarrhea in 39 out of 50 cases of acute infantile diarrhea (39%) and 38 out of 50 cases of persistent infantile diarrhea (38%).

Of the total 77 *E. coli* isolates, 15 generated positive results with multiplex PCR for two specific genes *aggR* and east. When these 15 PCR positive strains were studied for biofilm production, 12 (80%) strains showed positive results by quantitative microtitre plate assay. Raju and Ballal (2007) reported that of the total 100 *E. coli* isolates, 23 generated positive results with multiplex PCR for two specific genes *aggR* and *east*. Of which 20 (86%) strains showed positive results by quantitative microtitre plate assay. They also found that none of the 50 control samples collected showed positive results with biofilm. On the other hand, Helmi et al. (2010) showed that by quantitative biofilm assay out of total 300 *E. coli* isolates (200 cases and 100 controls) they could detect 65 EAEC strains (32.5 %). All controls showed negative results. This discrepancy may be attributed to different antimicrobial policy used in each community.

The results of this study showed that EAEC were detected in all age groups, especially in the less than 2 year age group; 12 cases (80%) less than two year, three cases (20%) more than 2 year. In accordance with these results, Helmi et al. (2010) showed that fifty five (84.5%) out of the 65 EAEC strains were isolated from patients below 24 months of age (15). The study also was in agreement with the study of Lima et al. (2000). Almost all patients (86%, positive EAEC) were under 24 months of age, suggesting the development of resistance against agents could be with increasing age.

The results of the study show that there was no significant difference in the presence of EAEC in both sexes, between rural and urban areas. Also, there was no significant difference in seasonal presence between EAEC positive and negative cases. On contrary to our results, Helmi et al. (2010) showed that the presence of diarrhea showed higher rates which was recorded in the months of June-August, than in the months of December-February. This might be attributable to the fact that most common bacterial pathogens causing acute diarrhea occur during summer, while most common viral pathogens occur during winter.

In the present study, EAEC detection rates were higher in infants that were not breastfed. Exclusive breast feeding was found to be significantly associated with lower presence of EAEC diarrhea. This observation was supported by the study of Ghosh et al. (2001) where 7 of the 109 infants harboring EAEC were breastfed, while the remaining 102 were on other feeding modes.

In this study, the infection with EAEC strains is associated with watery mucoid, bloody diarrhea, low grade fever and sometimes vomiting. The presence of fever, vomiting, dehydration or pus in stools did not differ significantly between EAEC positive and negative cases. The study of Helmi et al. (2010) showed that the nature of diarrhea was watery mucoid in 76.9%, versus bloody mucoid in 23.1% of patients. Adachi et al. (2002) stated that the clinical symptoms of EAEC infection vary from one study to another. Although not all EAEC infections result in symptomatic illness, most studies suggest that EAEC infection results in gastrointestinal disease. The most commonly reported symptoms are watery diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, and low grade fever. EAEC can cause both an acute and a persistent (>14 days) diarrheal illness. EAEC is associated with significant fluid loss and dehydration but a bloody stool is relatively infrequent Fran et al. (2011).

As regard the antimicrobial susceptibility profile of EAEC strains, the results of this study showed that the highest sensitivity of the isolated strains was to Amikacin (85.7 and 87.5%) and Cefoperazone (47.1 and 62.5%) then to Cefotrioxone (28.5 and 50.00%) and Imepenem (42.8 and 62.5%) in acute and persistent diarrhea and the lowest sensitivity was to sulphamethoxole/-trimethoprim (0.0 %) and Amoxicillin-Clavulanic (28.5 and 12.5%) in acute and persistent diarrhea. In accordance with these results, Paterson and Yu (1999) showed that the highest sensitivity of the strains of EAEC was to amikacin and ceftazidime and the lowest sensitivity was to ampicillin. Glandt et al. (1999) showed that EAEC-mediated diarrhea responded to therapy with ciprofloxacin and this was actually supported by the dissimilarities in the intestinal inflammatory markers seen in the ciprofloxacin- and placebo-treated populations. In another study, Sang et al. (1997) studied the association of multi-drug resistant EAEC isolated from persistent diarrhea in Kenyan children, and they found that EAEC was resistant to tetracycline, ampicillin, erythromycin, trimethoprim-sulphamethoxazole and amoxillin /-clavulanate. These discrepancies of the results of antimicrobial susceptibility profile of EAEC in the different studies may be due to the different policies of antibiotic therapy in different communities and this may be alarming for the importance of rapid and economic detection of EAEC in childhood diarrhea to improve the morbidity and mortality of the disease.

On another side, Sobieszczanska et al. (2003) showed

that many EAEC infections are self-limited. Symptomatic infections are usually treated empirically because laboratory diagnosis is not routinely available. EAEC susceptibility varies by region. In most regions, EAEC strains are susceptible to the fluoroquinolones, azithromycin, rifaximin, amoxicillin/clavulanic acid and nalidixic acid.

Conclusions

High incidence of EAEC associated diarrhea among pediatric cases in Egypt must be considered before decision of antimicrobial therapy. Quantitative biofilm assay is simple, rapid and convenient method for detection of EAEC in comparison with molecular methods and can therefore be recommended as a rapid screening test for EAEC in clinical laboratories.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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