

Evaluation of a multiplex PCR method to detect enteroaggregative *Escherichia coli*

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ABSTRACT: Enteroaggregative *Escherichia coli* (EAEC) has been implicated in sporadic diarrhea in children and adults and has been identified as the cause of several outbreaks worldwide. The HEp-2 test remains the gold standard for identification of this pathotype. A 60-65 MDa plasmid encodes the aggregative adherence fimbriae (AAF/I and AAF/II), a transcriptional activator (*aggR* gene), the enteroaggregative heat-stable enterotoxin EAST1 (*astA* gene) and a cytotoxin (Pet). The standard assay for EAEC is performed only in research laboratories, because it is expensive, labor intensive and time-consuming. The Polymerase Chain Reaction (PCR) offers the possibility of rapid diagnosis. In the current study, a multiplex PCR assay which checks *aggR* and *astA* genes was designed. Eighty-eight *E.coli* strains, isolated from children with acute diarrhea in Mendoza, Argentina, were characterized by the reference method (HEp-2 assay), and by *aggR-astA* PCR. A strong correlation between the presence of the specific marker *aggR* and the reference test was found. The *astA* gene had a similar distribution between aggregative and localized strains, indicating that this gene could not be considered as a marker of EAEC. We conclude that *aggR* may be used to identify EAEC, using the PCR method as a screening test.

Introduction

Epidemiological studies done in the past century have demonstrated an association of specific serotypes of *Escherichia coli* and diarrhea. Strains belonging to these serotypes were referred as Enteropathogenic *Escherichia coli* (EPEC), but early studies did not identify any pathogenic factor, that could account for their pathogenicity.

Since EPEC adherence to intestinal mucosa *in vivo* has been demonstrated, initial work attempted to char-

acterize adhesive mechanisms. In addition, the development of *in vitro* human-epithelial cell adherence tests demonstrated the correlation between some phenotype and the pathogenicity of EPEC. (Cravioto *et al.*, 1979; Cravioto *et al.*, 1991; Law, 1994; Nataro *et al.*, 1987).

With the HEp-2 or Hela cell assay three distinct patterns of adherence have been described: Localized Adherence (LA) in which bacteria form characteristic microcolonies on the surface of the HEp-2 cell (Fig. 1), Diffuse Adherence (DA), in which bacteria adhere evenly to the whole cell surface (Fig. 2), and aggregative adherence, in which aggregated bacteria attach to the cell in a stacked-brick arrangement (Fig. 3). Thus, this test allows to differentiate among all three adherent diarrheogenic categories: EPEC, Diffuse Adherence *Escherichia coli* (DAEC) and Enteroaggregative *Escherichia coli* (EAEC).

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The LA phenotype is associated to the induction of the attaching and effacing lesions (A/E) produced by EPEC. The most notable feature of the epidemiology of disease due to EPEC is the striking age distribution, seen in people infected with this pathogen. EPEC infection is primarily a disease of infants younger than 2 years, with a strongest correlation in infants younger than 6 months. In children older than 2 years, EPEC can be isolated from healthy and sick individuals, but a

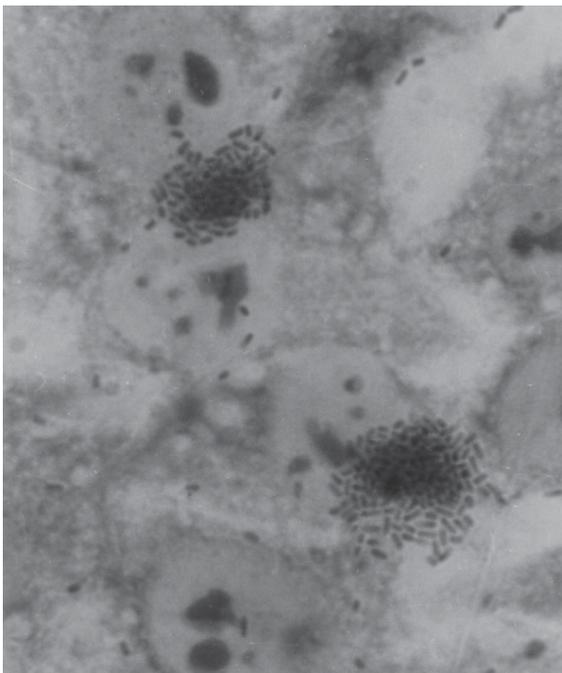


FIGURE 1. Localized Adherence (LA) typical of EPEC. Bacteria form characteristic microcolonies on the surface of the HEp-2 cell. (X 1,000)

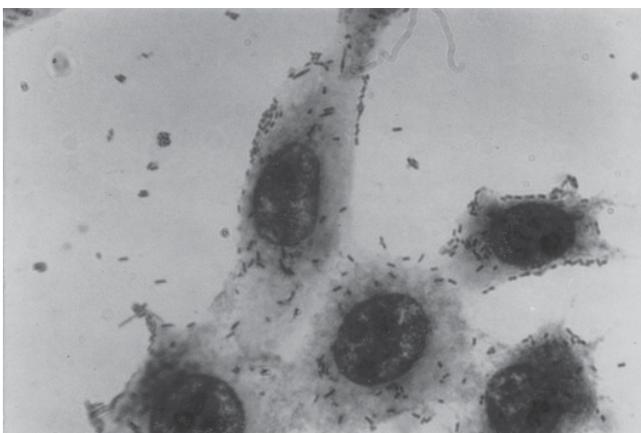


FIGURE 2. Diffuse adherence (DA) which defines DAEC. Bacteria are dispersed over the cell surface. (X 1,000)

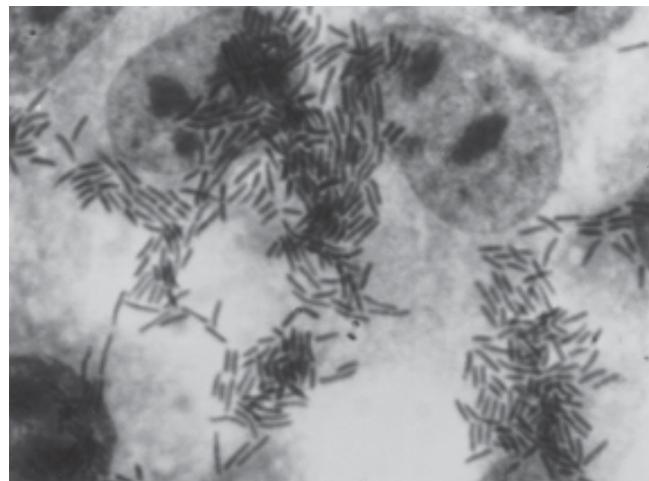


FIGURE 3. Aggregative Adherence (AA), which defines EAEC. Bacteria attach to both cells and coverslips in a stacked brick appearance. (X 1,000)

statistically significant correlation with disease is usually not found (Nataro and Kaper, 1998). The pathogenic role of *E. coli* showing a DA pattern (DAEC) in the etiology of diarrheal disease is controversial. EAEC is an increasing important cause of diarrhea. *E. coli* belonging to this category produces watery diarrhea, which is often persistent and can be inflammatory (Bhatnagar *et al.*, 1993).

EAEC has been implicated in sporadic diarrhea in children and adults and has been identified as the cause of several outbreaks worldwide (Bhan *et al.*, 1989; Cobeljic *et al.*, 1996; Nataro *et al.*, 1998; Okeke and Nataro, 2001). The HEp-2 test remains the gold standard for identification of this pathotype. In at least some strains, adherence is encoded on a 60-65 MDa plasmid (designated pAA) that also encodes several other putative virulence factors. pAA- encoded factors include the aggregative adherence fimbriae (AAF/I and AAF/II), a transcriptional activator (*aggR* gene), the enteroaggregative heat-stable enterotoxin EAST1 (*astA* gene) and a 104-k-Da cytotoxin designated as Pet (Czeczulin *et al.*, 1991; Eslava *et al.*, 1998; Baudry *et al.*, 1990; Debroy *et al.*, 1994).

The standard assay for EAEC, which demonstrates aggregative attachment of organisms to HEp-2 cells, is performed only in research laboratories, because it is expensive, labor intensive and time-consuming. With the advent of Polymerase Chain Reaction (PCR), it has become possible to identify pathogenic factors in bacterial isolates, offering the possibility of rapid diagnosis of *E. coli* infections (Dutta *et al.*, 1999; Okeke *et al.*,

2000; Schmidt *et al.*, 1995; Tsai *et al.*, 2003; Yatsuyanagi *et al.*, 2002; Yatsuyanagi *et al.*, 2003).

In a previous study, we evaluated the value of a PCR method as a screening test for the diagnosis of EAEC strains (Rüttler *et al.*, 2002). The virulence factors investigated in that study were AAF/I and EAST1. The sensitivity obtained for AAF/I was low, suggesting that some of the strains possessed other adherent factor, which in the present work was not investigated.

In the current study, we designed a set of primers to identify *aggR* gene, which contains the sequence for a transcriptional activator common to both AAF/I and AAF/II, in order to improve the sensitivity previously obtained, when were only checked AAF/I. We included both, *aggR* and *astA* gene (EAST1) in a multiplex PCR assay (Elías *et al.*, 1999; Nataro and Cravioto, 1998; Rich *et al.*, 1999; Nataro *et al.*, 1993).

Materials and Methods

The strains examined in this work were isolated during an epidemiologic study of acute diarrhea in children younger than 2 years old. The present study was performed in the Humberto Notti Children's Hospital in Mendoza, Argentina, during 1999 and 2000.

Samples were obtained by spontaneous evacuation, collected in sterile recipients and processed within two hours. Direct microscopic exam was performed on the stool with Gram stain, Blue Methylene stain (leukocytes observation) and fresh exam in order to detect *Entamoeba histolytica* and *Giardia lamblia*. To identify cryptosporidium, modified Ziehl – Neelsen stain was performed. *Shigella* spp., and *Salmonella* were also investigated by standard methods. An adequate stool dilution was cultivated on Petri plates with Mac Conkey agar. From each plate three lactose fermentative colonies were taken and standard biochemical tests were performed on them.

A total of 88 strains, biochemically identified as *Escherichia coli*, were analysed in the present study.

Serotyping

Identification of the somatic (O) antigens of the strains was done by the standard agglutination method, using monovalent serum provided by the Malbrán Institute (Bs.As., Argentina). The investigated serogroups were the following: O26, O44, O55, O86, O111, O114, O119, O125, O126, O127, O128, O136, O142.

Tissue culture adhesion tests in HEp – 2 cells

Adhesion tests were performed in HEp-2 cells (ATCC: American Type Culture Collection), acquired from the Argentinean Cell Bank Association (ABAC). Cells were cultured in Minimum Essential Medium (MEM), with Earle salts (Sigma Co.), supplemented with 10% bovine fetal serum (Gen, Bs. As., Argentina), without antibiotics, in a stove at 37°C and 5% CO₂. The method was adapted from Cravioto *et al.* in 1979. The HEp-2 cells were grown to 50-70% confluent monolayers on glass coverslips in tissue culture plates. After a gentle wash with phosphate-buffered saline (PBS), 1 mL of fresh MEM containing 2% fetal bovine serum and 0.5% D-mannose were added to each well, with 20 µL (2x10⁵) of an overnight *E.coli* broth culture. The mixture was incubated for 3 h at 37°C in 5% CO₂. After the incubation period, cells were washed several times with PBS, fixed with 70% methanol, stained with 10% Giemsa stain for 15 min and examined by light microscopy (100X). The reference strains used were: 2348/69: localized pattern, AA17-2 (O3:H2): aggregative pattern, RS 51-1: diffuse pattern, HS (O9:H4): negative adherence pattern. Samples were analyzed in duplicate to eliminate potential technical errors. Discordant pair results were repeated in duplicate, using the same technique.

Multiplex PCR

The *E.coli* strains were examined by Multiplex PCR to detect the genes *astA* (which codify EAST1-Enterotoxigenic Heat Stable Enterotoxin I) and *aggR* (Transcription Regulator for AAF/I and AAF/II). This reaction, which included *primers* for both genes, allowed the detection of *E. coli* that were bearing one or both characteristics.

For the PCR reaction, DNA was obtained from the sample in process. Bacteria were harvested from 1 mL of an overnight culture in TSB, suspended in 150 µL of Triton 1% on buffer TE, on an 1.5 mL-ependorf tube. The tube was boiled for 10 min and centrifuged at 10,000 rpm. The supernatant was used as a template for the PCR reaction.

The *primers* for the amplification of a 108 bp of the *astA* gene were:

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START 5' GCC ATC ACA GTA TAT CCG 3'
STOP 5' GCG AGT GAC GGC TTT GTA GT 3'
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Nucleotide sequences corresponding to the fimbriae genes I and II from EAEC (AAF/I and AAF/II) were obtained. The expression of both fimbrial structures is regulated by a common gene, denominated *aggR* (transcription regulator gene). Thus, the design of specific *primers* for this gene, would allow to detect both types of fimbriae in only one reaction. The PC Gene Programm was used for the search of the right *primers*. From a variable number of selected *primers* by the program, one pair was chosen, which flanks a 430 nucleotides region:

START 5' AGA CGC CTA AAG GAT GCC C 3'
STOP 5' GAG TTA TCA AGC AAC AGC AAT GC 3'

The sequence of both *primers* was introduced on the GenBank data base in order to compare the sequence of all the incorporated genes. The result showed 100% homology with the *aggR* gene, access number Z32523, assuring that it would only amplify the desired gene.

The PCR mixture contained 10 mM Tris-HCl (pH 9 at 25°C), 50 mM KCl and 0.1% Triton® X-100, MgCl₂ 1.5 mM, dNTP 0.2 mM each, 2 U of Taq polymerase (Promega), 1 µM each primer, water and 10 µL DNA.

The PCR program was: one step of 5 min at 94°C, 30 cycles of: 30 s at 94°C, 1 min at 57°C, 40 s at 72°C, one step of 5 min at 72°C.

AA17/2 (O3:H2) strain was used as the positive control, and HS (O9:H4) as the negative one. The strains were from the collection of the Center for Vaccine Development (Maryland, USA) (Fig. 4).

Statistical analysis

The sensitivity and specificity values were obtained by comparison between the HEP-2 assay and the PCR using Fisher exact test. A *P* value of < 0.05 was considered statistically significant.

Results

Based on the adhesion to HEP-2 cells, 34 strains (39%) showed the localized (LA) pattern; 23 strains (26%) were aggregative (AA) and 13 strains (15%) were diffuse. Eighteen strains (20%) did not show any adherence pattern. Figure 5 shows the results of the HEP-2 assay.

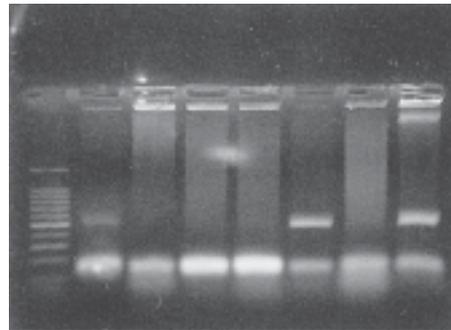


FIGURE 4. Agarose gel electrophoresis of PCR amplified DNA products. Lane 1 molecular size marker (100-bp ladder; Promega); lanes 3, 4, 5 and 7, *astA* PCR(108 bp); lanes 2, 6 and 7, *astA* and *aggR* PCR (108-bp and 430-bp respectively).

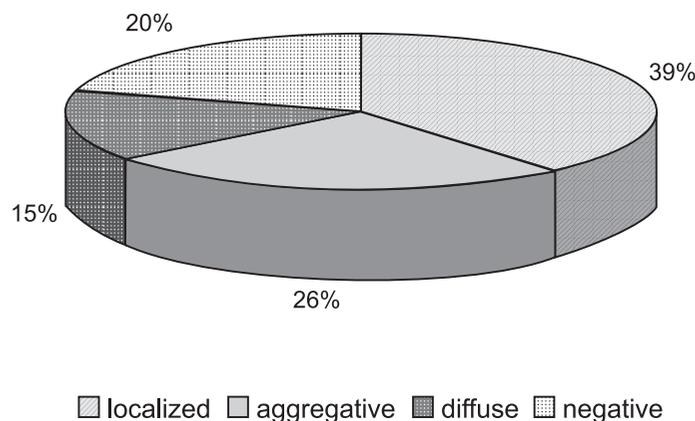


FIGURE 5. Distribution of adherence patterns.

Table 1 shows the results for *aggR* PCR and aggregative adherence pattern by HEp-2 test.

Twenty of the 23 aggregative pattern strains harbored the *aggR* gene, according to the PCR results, improving the sensitivity previously obtained.

Table 2 compares the results of *astA* PCR and HEp-2 assay. Although 57 strains harbored the *astA* gene, only 16 of these expressed the AA pattern in the HEp-2 assay. Seven strains that presented the AA phenotype in the HEp-2 assay were negative for the *astA* gene. The association between both methods was not significant.

TABLE 1.

Comparison of results with *aggR* PCR and aggregative adherence pattern by HEp-2 test

N° of strains	Percentage	<i>aggR</i> PCR	Aggregative Adherence
20	23	Positive	Positive
3	3	Negative	Positive
3	3	Positive	Negative
62	70	Negative	Negative

95% Confidence Interval, Sensitivity: 87% (66-97%), Specificity: 95% (87-99%), Positive Predictive Value: 87% (66-97%), Negative Predictive Value: 95% (87-99%), Likelihood Ratio: 18.84, Fisher's exact test P < 0.0001

TABLE 2.

Comparison of results with *astA* PCR and aggregative adherence pattern by HEp-2 test

N° of strains	Percentage	<i>astA</i> PCR	Aggregative Adherence
16	18	positive	positive
7	8	negative	positive
41	47	positive	negative
24	27	negative	negative

95% Confidence Interval, Sensitivity: 70% (47-87%), Specificity: 37% (25-50%), Positive Predictive Value: 28% (17-42%), Negative Predictive Value: 77% (59-90%), Likelihood Ratio: 1.10, Fisher's exact test P = 0.6216.

TABLE 3.

Comparison of results with *astA* PCR and localized adherence pattern by HEp-2 test

N° of strains	Percentage	<i>astA</i> PCR	Localized Adherence
19	22	Positive	positive
16	18	Negative	positive
38	43	Positive	negative
15	17	Negative	negative

95% Confidence Interval, Sensitivity: 54% (37-71%), Specificity: 28% (17-42%), Positive Predictive Value: 33% (21-47%), Negative Predictive Value: 48% (30-67%), Likelihood Ratio: 0.76, Fisher's exact test P = 0.11

The *astA* gene have been detected not only in EAEC, but also in EPEC. Table 3 shows the comparison between this gene and the LA pattern, characteristic of EPEC. The *astA* gene was found in 57 (65%) of the strains, 19 (22%) of which showed the LA pattern. In addition 38 of the *astA* carrying strains presented another pattern of adherence. The serogroups and the adherence patterns of the *aggR* strains are described in Table 4. Among the serogroups found, O86, O44, O127 and O111 are epidemiologically related to EAEC; the rest belongs to Class I classical EPEC. The majority of *aggR* strains (78%) showed the aggregative pattern, three were diffuse and two negative.

Discussion

EAEC is a diarrheal pathogen of emerging importance, and it has been involved in both endemic pediatric diarrhea and outbreaks worldwide (Valentiner-Branth *et al.*, 2003; Dutta *et al.*, 1999). However, the pathogenic mechanisms of EAEC infection are not fully understood. Moreover, it appears to be a significant heterogeneity of virulence markers among EAEC isolates (Nataro *et al.*, 1995; Zamboni *et al.*, 2004). The specific role of the virulence determinants in the pathogenicity of diarrhea is being investigated. The demonstration of aggregative attachments of organisms to human epithelial cells (HEp-2) remains as the gold standard assay for EAEC. This test requires specialized facilities, and it can only be conducted in research laboratories (Miqdady *et al.*, 2002). The PCR offers new possi-

TABLE 4.

Serogroups and adherence patterns in *aggR* positive strains

N° strain	Serogroup	Adherence pattern
66	O125	aggregative
67	O125	aggregative
68	O55	aggregative
69	O86	diffuse
70	O44	negative
71	O125	aggregative
72	O55	aggregative
73	O28	aggregative
74	O55	aggregative
75	O28	aggregative
76	O126	aggregative
77	O55	aggregative
78	O55	aggregative
79	O114	diffuse
80	O126	diffuse
81	O125	aggregative
82	O55	aggregative
83	O119	aggregative
84	O55	aggregative
85	O119	aggregative
86	O136	aggregative
87	O127	aggregative
88	O111	negative

bilities for the diagnosis because it is sensitive, specific, practical and has lower cost than the cell culture assay.

This study was designed to evaluate a multiplex PCR method for identification of EAEC. The virulence markers included in the PCR were *aggR* and *astA*, because both are widely distributed among EAEC strains (Nataro *et al.*, 1994; Yoshikazu *et al.*, 2002).

The distribution of the different adherence patterns varies between different reports (Cravioto *et al.*, 1991; Nataro and Kaper, 1998; Okeke *et al.*, 2000; Ortiz *et al.*, 1998; Pabst *et al.*, 2003; Yatsuyanagi *et al.*, 2002). In this work, this distribution was similar to a previous study (Rüttler *et al.*, 2002), suggesting that the strains recovered from infected patients in our community are endemics. Studies to determine their

epidemiologic and clonal relationship are being conducted in our laboratory.

Among the 23 strains adhering to HEp-2 cells with AA phenotype, twenty harbored sequences homologous to the *aggR* gene, according to the PCR results, demonstrating a high concordance between both methods. Other authors obtained similar results when examined the relationship between the possession of EAEC plasmid-borne gene *aggR* and the ability of *E.coli* strains to adhere to HEp-2 cells (Jiang *et al.*, 2002; Tsukamoto, 1996).

Moreover, Tsukamoto (1996) proposed a PCR method based on the *aggR* gene; this method was shown to have greater sensitivity than the probe assay in detecting enteroaggregative *Escherichia coli*.

With respect to the *astA* gene, we did not find significant association with the aggregative phenotype in the HEp-2 assay. In fact, although 57 strains harbored the *astA* gene, only 16 of these expressed the AA pattern in the HEp-2 assay (Table 2).

The *astA* gene was initially identified in EAEC as a structural gene that encodes a distinct low-molecular-weight putative enterotoxin (Savarino *et al.*, 1993). In the last years, the *astA* gene has been detected not only in EAEC but also in EPEC, atypical EPEC, ETEC, STEC and EIEC strains. We also compared the *astA* PCR with the LA pattern, characteristic from EPEC. An interesting finding of this work was that the *astA* gene had a similar distribution between aggregative pattern (18%) and localized pattern (22%) strains, indicating that this gene would not be considered as a marker of EAEC. Some studies proposed the denomination of EAST1EC (Enteroggregative Stable Toxin *Escherichia coli*) for the strains which presented *astA* gene as the only factor, because reports suggesting the diarrheagenicity of EAST1 gene-possessing *E.coli* have gradually increased (Nishikawa *et al.*, 2002; Zhou *et al.*, 2002; Yatsuyanagi *et al.*, 2002). Further studies would be necessary to clarify the importance of the *astA* gene as putative diarrheagenic factor.

The serogroups and the adherence patterns of the *aggR* strains are described in Table 4. Between the serogroups founded, O86, O44, O127 and O111 are epidemiologically related to EAEC, the rest belongs to Class I classical EPEC. According to this findings, the serogroup of *E.coli* is not likely to correspond to the pathogenic factors.

The sensitivity and specificity of *aggR* PCR were 78% and 95%, respectively. Compared with our previous results, the sensitivity was higher without modification of the specificity. The latter allows us to affirm that

this method can detect more aggregative strains because the new gene is common to AAF/I and AAF/II strains.

Moreover, since in our study a strong correlation between the presence of the specific marker *aggR* and the reference test was found, we can conclude that *aggR* may be used to identify EAEC, using the PCR method as a screening test for the diagnosis of EAEC.

Since some strains that did not carry *aggR* gene adhered to HEp-2 cells with an aggregative pattern and could be pathogens, the adhesion assay should be used to complement the diagnosis in the absence of other known pathogen or in persistent cases of diarrhea.

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