

RESEARCH ARTICLE

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Detection and genetic analysis of the enteroaggregative *Escherichia coli* heat-stable enterotoxin (EAST1) gene in clinical isolates of enteropathogenic *Escherichia coli* (EPEC) strains

Lucas EP Silva¹, Tamara B Souza¹, Neusa P Silva² and Isabel CA Scaletsky^{1*}**Abstract**

Background: The enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) encoded by *astA* gene has been found in enteropathogenic *E. coli* (EPEC) strains. However, it is not sufficient to simply probe strains with an *astA* gene probe due to the existence of *astA* mutants (type 1 and type 2 SHEAST) and EAST1 variants (EAST1 v1-4). In this study, 222 EPEC (70 typical and 152 atypical) isolates were tested for the presence of the *astA* gene sequence by PCR and sequencing.

Results: The *astA* gene was amplified from 54 strains, 11 typical and 43 atypical. Sequence analysis of the PCR products showed that 25 strains, 7 typical and 18 atypical, had an intact *astA* gene. A subgroup of 7 atypical strains had a variant type of the *astA* gene sequence, with four non-synonymous nucleotide substitutions. The remaining 22 strains had mutated *astA* gene with nucleotide deletions or substitutions in the first 8 codons. The RT-PCR results showed that the *astA* gene was transcribed only by the strains carrying either the intact or the variant type of the *astA* gene sequence. Southern blot analysis indicated that *astA* is located in EAF plasmid in typical strains, and in plasmids of similar size in atypical strains. Strains carrying intact *astA* genes were more frequently found in diarrheic children than in non-diarrheic children ($p < 0.05$).

Conclusion: In conclusion, our data suggest that the presence of an intact *astA* gene may represent an additional virulence determinant in both EPEC groups.

Keywords: EAST1 gene, *astA* gene, Enteropathogenic *Escherichia coli*

Background

Enteropathogenic *Escherichia coli* (EPEC) are an important cause of infant diarrhea in developing countries [1]. The majority of EPEC isolates belong to classic serotypes derived from 12 classical O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158) [2,3]. EPEC induces attaching and effacing (A/E) lesions on epithelial cells, characterized by microvilli destruction, cytoskeleton rearrangement, and the formation of a pedestal-like structure at the site of bacterial contact [4]. The A/E genes are localized to the locus

for enterocyte effacement (LEE) and encode intimin, a type III secretion system, secreted proteins and the translocated intimin receptor [5-7].

“Typical” EPEC strains (tEPEC) contain also the EPEC adherence factor (EAF) plasmid [8], which carries genes encoding a regulator (*per*) [9] and the bundle-forming pili (BFP) [10]. EPEC strains lacking the EAF plasmid have been designated “atypical” EPEC (aEPEC) [11]. Recent epidemiological studies indicate that aEPEC are more prevalent than tEPEC in both developed and developing countries [1]. Some aEPEC strains are genetically related to the enterohemorrhagic *E. coli* (EHEC), and both are considered as emerging pathogens [12].

Typical EPEC strains express only the virulence factors encoded by the LEE region and the EAF plasmid, with

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the exception of the cytolethal distending toxin produced by O86:H34 strains and the enteroaggregative heat-stable enterotoxin 1 (EAST1) found in O55:H6 and O127:H6 strains. In contrast, aEPEC strains frequently express EAST1 and additional virulence factors not encoded by LEE region [12]. In a previous study [13], EAST1 was the most frequent (24%) virulence factor found in a collection of 65 aEPEC strains, and was significantly associated with children diarrhea.

EAST1-positive aEPEC strains have been associated with outbreaks of diarrhea involving children and adults in the United State [14] and Japan [15]. However, it is not sufficient to simply probe strains with an *astA* gene probe due to the existence of EAST1 variants [16]. In one study, 100% of the O26, O111, O145, and O157:H7 enterohemorrhagic *E. coli* (EHEC) strains examined carried DNA sequences homologous to the EAST1 gene (SHEAST) with two different mutation types. Type 1 SHEAST has 12 nucleotide non-synonymous substitutions including one in the initiation codon; type 2 SHEAST lacks the first 8 codons of EAST1 sequence [16]. The focus of the study was to investigate the *astA* gene sequence present in tEPEC and aEPEC strains. The strains were collected in different cities of Brazil in different periods of time and in a previous

study poor relatedness was observed by RAPD analysis of 118 strains belonging to this collection [13].

Results and discussion

We examined 222 EPEC strains (70 typical and 152 atypical) for the presence of the *astA* gene by PCR using primers that anneal to the 5' ends of the EAEC 042 *astA* gene sequence [16]. Those strains were isolated from diarrheic and non diarrheic Brazilian children in previous studies [17-20]. As shown in Table 1, 11 (16%) tEPEC and 43 (28%) aEPEC strains were positive in the PCR assay. Among the aEPEC PCR-positive strains, 13 belonged to the O26 and O119 serogroups.

The 54 *astA* gene PCR products were sequenced. Twenty five strains, 7 tEPEC and 18 aEPEC, carried the DNA sequence identical to the EAST1 gene (042-type EAST1) (Figure 1). A subgroup of 7 aEPEC strains presented a variant type of the 042-type EAST1 gene sequence, with four non-synonymous nucleotide substitutions. Nine other strains, including one typical, carried either the sequence identical to type 1 SHEAST (7 strains) or to type 2 SHEAST (two strains). The remaining 13 strains carried mutated sequences of the 042-type EAST1 (five strains), type 1 SHEAST (two strains) or type 2 SHEAST (six strains) genes.

Table 1 EPEC-*astA* strains isolated from diarrheic and non-diarrheic children

EPEC	Serotype	No. of strains (positive/total)		
		Diarrheic children	Non-diarrheic children	Total of children
tEPEC	O55:NM;HND	0/13	0/1	0/14
	O86:NM;H34	0/2	0	0/2
	O111:NM;H2;HND	4/9	0	4/9
	O119:NM;H6;HND	2/22	0/3	2/25
	O127:NM;H6	0/1	2/3	2/4
	Other serotypes ^a	3/14	0/2	3/16
Subtotal		9/61	2/9	11/70
aEPEC	O26:H11;HND	6/10	0/2	6/12
	O55:HND	2/3	1/2	3/5
	O111:NM	2/2	1/2	3/4
	O114:NM	0	0/1	0/1
	O119:H2;HND	7/9	0/3	7/12
	O126:NM	0/1	0	0/1
	O127:NM;H40	0/3	0/1	0/4
	O128:NM	0/3	0	0/3
	O142:NM;H2	1/8	0	1/8
	Other serotypes ^b	18/68	5/34	23/102
Subtotal		36/107	7/45	43/152
Total		45/168	9/54	54/222

^aO2:H2;H45; O101:H33; O145:HND; O157:HND; O162:H33; ONT:H45; ONT:HND.

^bO4:HND; O15:HND O33:H6; O35:H19; O37:HND; O49:HND; O61:HND; O63:HND; O79:HND; O85:H40; O96:HND; O98:HND; O101:NM; O103:NM; O105:H7; O108:H31; O109:H54; O117:HND; O132:HND; O141:HND; O1523H2; O156:H16; O157:HND; O167:H6; O169:H6; O175:HND; ONT:NM; ONT:H18; ONT:HND.

Note: NM, nonmotile, ND, nondetermined, ONT, nontypeable.

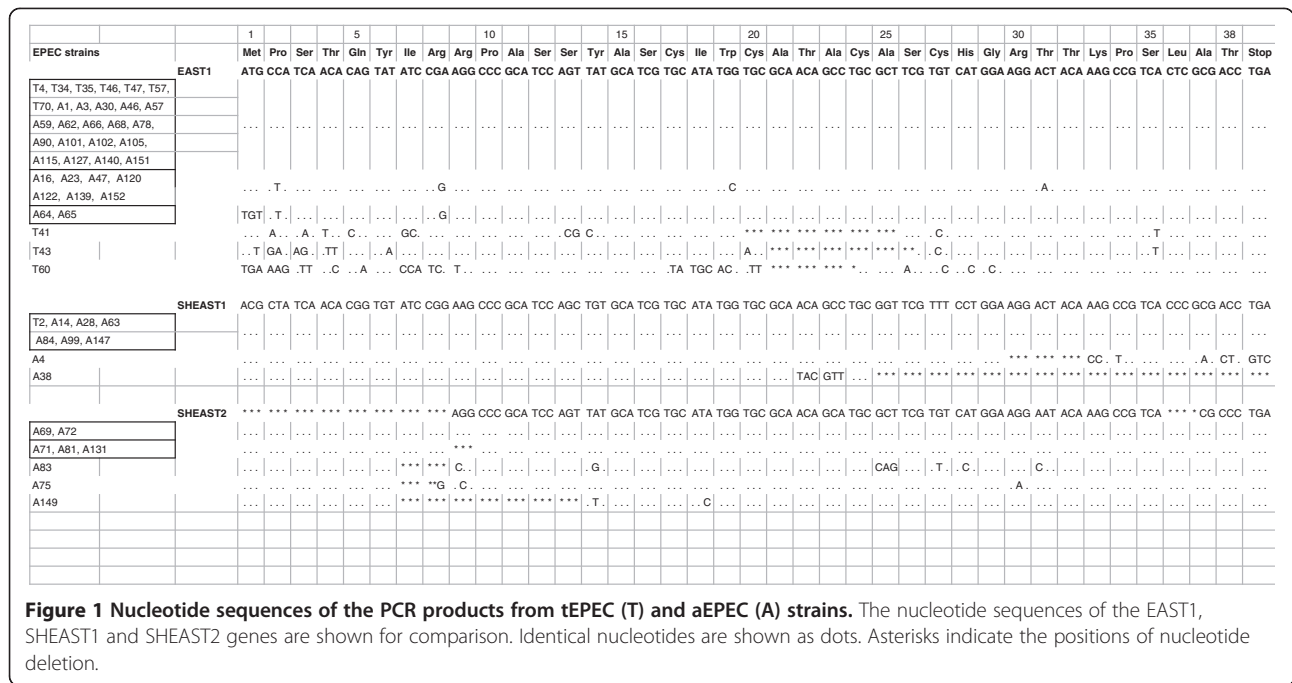


Figure 1 Nucleotide sequences of the PCR products from tEPEC (T) and aEPEC (A) strains. The nucleotide sequences of the EAST1, SHEAST1 and SHEAST2 genes are shown for comparison. Identical nucleotides are shown as dots. Asterisks indicate the positions of nucleotide deletion.

The expression of EAST1 was examined by RT-PCR and quantitative RT-PCR. The RT-PCR results showed that the *astA* gene was transcribed only by the strains carrying either the intact or the variant type of the *astA* gene sequence (Figure 2). The *astA* gene expression levels of the 32 RT-PCR positive strains (CT values ranged from 20.3 ± 0.11 to 21.6 ± 0.04) were nearly identical to that of EAEC 042 strain (CT value 20.8 ± 0.01).

Plasmids of the 54 PCR-positive strains were examined for *astA* gene presence by Southern blot hybridization with the *astA* probe. In 23 (42.6%) strains, a single copy

of the *astA* gene was located to a large plasmid (Figure 3). In all the eleven tEPEC strains, the *astA* probe hybridized to the EAF plasmid as previously reported [21], and in twelve aEPEC the *astA* probe hybridized with large plasmids of similar size. The plasmids of the remaining strains were *astA* probe negative.

We previously reported that 24% of 65 aEPEC strains hybridized with a DNA probe for EAST1 [13]. Here, we analyzed by PCR a larger group of EPEC, including typical strains and found that 11 (16%) of 70 tEPEC and 43 (28%) of 152 aEPEC were *astA* positive. Sequence analysis of the

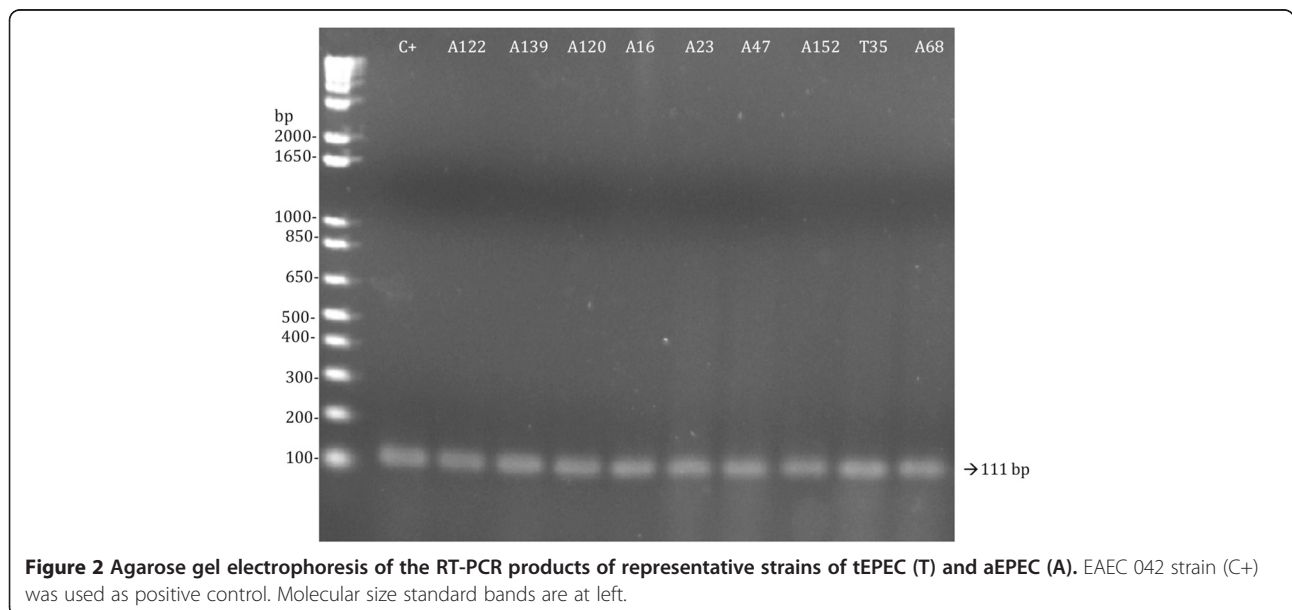
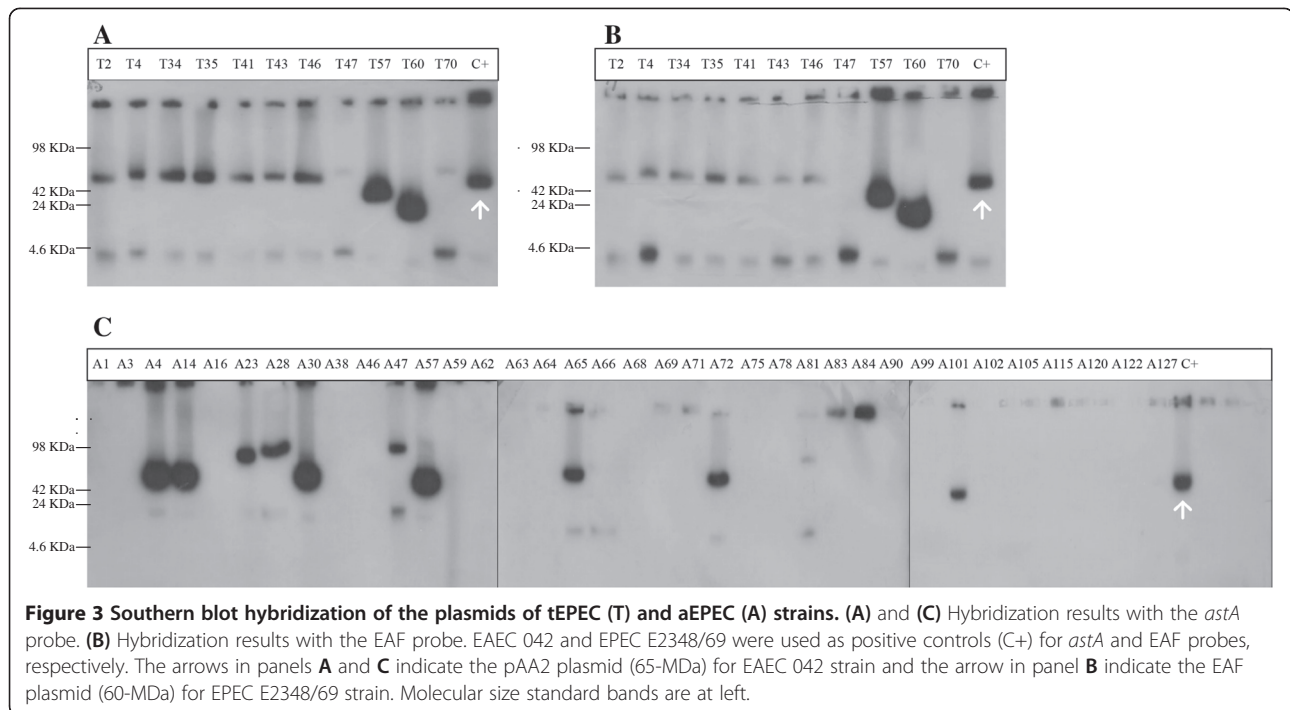


Figure 2 Agarose gel electrophoresis of the RT-PCR products of representative strains of tEPEC (T) and aEPEC (A). EAEC 042 strain (C+) was used as positive control. Molecular size standard bands are at left.



PCR products showed that 7 (63.6%) of 11 tEPEC and 18 (41.9%) of 43 aEPEC had an intact 042-type *astA* gene.

As shown in Table 2, strains carrying intact *astA* gene were more frequently found in diarrheic children than in non-diarrheic children ($p = 0.03$, Fisher's exact test). However, we should point out that among the 222 strains analyzed only 118 were collected from a case-control study [13].

The EAST1 gene family includes one major type of sequence, i.e. the *astA* of EAEC strain 042 that is widely distributed among different diarrheogenic *E. coli* strains [21-26] and four variant types of EAST1, i.e. the EAST1v1 of EAEC 17-2 [21,22], EAST1v2 of EPEC N1 [21], and EAST1v3 and EAST1v4 of *E. coli* O166:H15 [25].

In this study, a subgroup of aEPEC strains had a new variant type of EAST1 gene sequence that differed from those previously reported, and was denominated EAST1v5

(Figure 4). The RT-PCR analysis showed that EAST1v5 was transcribed to produce mRNA. However, more studies are necessary to determine whether EAST1v5 is associated with a functional polypeptide toxin.

Conclusion

In conclusion, our data suggest that the presence of an intact *astA* gene may represent an additional virulence determinant in both EPEC groups.

Methods

Bacterial strains

The 222 EPEC strains examined in this study included 176 strains isolated in 1999 to 2004 during an epidemiological study of acute diarrhea in children <2 years of age conducted in different regions of Brazil, and 46 strains isolated from children <5 years of age with diarrhea in

Table 2 Sequences of the *astA* gene found in EPEC strains isolated from diarrheic and non-diarrheic children

<i>astA</i> gene sequence type	N (%) of strains from:		Serogroup (n)
	Diarrheic children	Non-diarrheic children	
042-type EAST1	24 (14.3)	1 (1.8) ^a	O9 (1), O33 (2), O108 (2), O111 (1), O119 (8), O142 (1), O152 (1), O157 (1), O169 (1), OND (7)
EAST1v5	6 (3.6)	1 (1.8)	O26 (1), O9 (1), O96 (1) O111 (1), O141 (1), ONT (2)
type 1 SHEAST	6 (3.6)	1 (1.8)	O26 (1), O55 (1), O103 (1), O153 (1), OND (3)
type 2 SHEAST	2 (1.2)	0	O26 (1), O55 (1)
mutant	7	6	O26 (3), O55 (1), O111 (5), O119 (1), O127 (2), ONT (1)
Total	45	9	

^a $p = 0.03$; Fisher's exact test (diarrheic x non-diarrheic).

Nucleotide sequence and accession number

The EAST1v5 gene sequence was deposited in the NCBI database under accession number KJ47188.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LEPS and TBS performed experiments and analyzed data. NPS and ICAS wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This study was supported by research grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank Dr. Renata Torres de Souza for her help with the nucleotide sequence deposition.

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Received: 29 January 2014 Accepted: 23 May 2014

Published: 30 May 2014

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doi:10.1186/1471-2180-14-135

Cite this article as: Silva et al.: Detection and genetic analysis of the enteroaggregative *Escherichia coli* heat-stable enterotoxin (EAST1) gene in clinical isolates of enteropathogenic *Escherichia coli* (EPEC) strains. *BMC Microbiology* 2014 **14**:135.