EPIDEMIOLOGY OF INFECTION

A case-control study of diarrhoea in children caused by *Escherichia coli* producing heat-stable enterotoxin (EAST-1)

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Escherichia coli strains associated with diarrhoeal disease have been classified into several types according to the pathogenic mechanism. Among these, enteroaggregative *E. coli* strains (EAggEC) have been associated with persistent childhood diarrhoea. Some strains of EAggEC produce a heat-stable toxin (EAST-1) that differs from others described previously. The main goal of this case-control study was to determine the prevalence of EAggEC and EAST-1-producing *E. coli* strains as a cause of diarrhoea in children in Spain and to study their in-vitro susceptibility to 21 antimicrobial agents. In the case group (115 children) 22 (19%) isolates and four (3.5%) isolates were EAST-1-producing *E. coli* and EAggEC, respectively, whereas in the control group (79 children) four (5%) isolates produced EAST-1 ($p = 0.005$) and three (3.8%) isolates were EAggEC. The present study suggests that EAST-1-producing *E. coli* strains are associated with diarrhoeal diseases in Spanish children, whereas EAggEC strains are not. Moreover, EAST-1-producing *E. coli* strains showed a high susceptibility to all the antimicrobial agents tested except for ampicillin.

Introduction

The aetiology of diarrhoea in infants and children is quite different in developing and developed countries; in Spain *Campylobacter* spp. are the most common cause [1]. However, in general, 40–50% of cases remain undiagnosed microbiologically. At least six different diarrhoeagenic types of *Escherichia coli* have been identified. These include: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), verotoxin-producing *E. coli* (VTEC) some strains of which are enterohaemorrhagic (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC). EAggEC is associated with persistent diarrhoea in children [2] and it has been identified by a tissue culture adherence assay, where it presents an aggregative pattern, characterised by the fact that bacteria adhere to each other and appear as stacked-brick clumps on the HEP-2 cell [3]. Although EAggEC has been recognised as an important cause of acute and persistent diarrhoea in children in developing countries [2, 4], few studies

have sought the presence of EAggEC in children in developed countries [5, 6]. Some strains of EAggEC produce a heat-stable enterotoxin (EAST-1) different from other *E. coli* heat-stable enterotoxins, although it exhibits some functional relatedness to the heat-stable toxin STa of ETEC [7]. Recently, it has been shown that EAST-1 production is not restricted to enteroaggregative *E. coli* [8]. The main aim of this case-control study was to define the association of EAggEC or EAST-1-producing *E. coli* with diarrhoea in children < 5 years old in Spain and to analyse the susceptibility to 21 antimicrobial agents.

Materials and methods

Specimens

Stool specimens were collected from children with diarrhoea who attended the outpatients' clinic of the Sant Joan de Deu Hospital, a paediatric hospital in Barcelona, Spain. Diarrhoea was defined as three or more stools per day for at least 3 days with a decrease in the consistency to an unformed state. The control group consisted of children who attended the hospital for non-diarrhoeal illness and who had not had diarrhoea in the previous 2 weeks. The samples were

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processed by conventional methods [9]. All the samples in which other enteropathogens were detected were discarded from the study. Therefore, the study was started with 115 *E. coli* isolates from the case group and 79 from the control group.

PCR to detect EAggEC and EAST-1-producing *E. coli*

Two colonies of *E. coli* were picked from MacConkey agar and a PCR technique was used to identify the enteroaggregative characteristics and heat-stable toxin production. The two primers described by Schmidt *et al.* [10] were used to detect enteroaggregative *E. coli*. Two oligonucleotide primers were designed, based on the nucleotide sequence described by Savarinol *et al.* [7] to identify EAST-1 positive strains. The primers, 5'ATGCCATCAACACAGTATAT3' and 5'GCGAGT-GACGGCTTTGTAGT3' were used to amplify the fragment of *astA* gene, which codes for EAST-1, from nucleotides 132–241. Briefly, half a colony of each isolate was suspended in 25 µl of distilled sterile water and boiled for 10 min. After a short centrifugation 25 µl of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM KCl, 3.0 mM MgCl₂, gelatin 1% w/v, 400 µM dNTPs and 1 µM of the appropriate primers, together with 2.5 Units of *Taq* polymerase (Gibco-BRL) were added. The mixture was overlaid with oil and amplified with the following PCR programme: 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; with a single final extension of 72°C for 5 min. Amplified DNA products were resolved by electrophoresis in agarose 2% w/v gels containing ethidium bromide 0.5 mg/L. The PCR product was extracted from the agarose gel and the DNA was sequenced with the *Taq* DyeDeoxiTerminator Cycle Sequencing kit and analysed in an automatic DNA sequencer (373A; Applied Biosystems).

Susceptibility testing

The susceptibility to antimicrobial agents was determined with an automated microdilution system (Microscan Walkaway, Baxter, Co. West Sacramento, CA, USA).

Results and discussion

By an assay with cultured HEP-2 or HeLa cells diarrhoeagenic *E. coli* strains may be classified on the basis of which adherence pattern they show: localised, diffuse or aggregative. However, discrepancies among different studies could be caused by the subjective interpretation of the adherence test, as well as to technical differences in the cell adherence procedure [11]. Therefore, the PCR technique described by Schmidt *et al.* [10] was used to detect EAggEC strains in this study and specific primers were designed to detect the *astA* gene. Both PCR products

had the expected size of 109 bp corresponding to the amplified region of the *astA* gene and 630 bp corresponding to the amplified region of the *aggA* gene (Fig. 1). The purified product of the *astA* gene was directly processed once for DNA sequencing and the sequence obtained from the amplified region was exactly the same as that previously described by Savarino *et al.* [11]. A very good correlation between the PCR technique for detecting enteroaggregative *E. coli* and the adherence test has been shown [12].

Table 1 indicates the frequency of isolation of EAggEC and EAST-1-producing *E. coli* from diarrhoeic and non-diarrhoeic patients. EAggEC was isolated from 3.5% (4 of 115) of children with diarrhoea compared with 3.8% (3 of 79) children without diarrhoea ($p = 0.308$), whereas EAST-1-producing *E. coli* was isolated from 19% (22 of 115) of children with diarrhoea compared with 5% (4 of 79) of children without diarrhoea ($p = 0.005$). Only one of the four EAggEC isolates from the case group showed production of EAST-1. This result is in contrast to that presented by Savarino *et al.* [8] who found that 41%

Fig. 1. Agarose gel electrophoresis of DNA fragments amplified with specific primers at *ast* gene (1), and to detect EAggEC (2), M, mol. wt marker (Boehringer).

Table 1. Enteroaggregative *E. coli* and EAST-1-producing *E. coli* from children with and without diarrhoea detected by PCR

<i>E. coli</i>	Number of children		p value*
	Cases (n = 115)	Controls (n = 79)	
Heat-stable toxin producing	22	4	0.005
Enteroaggregative	4	3	0.308

*Fisher's exact test

of 227 EAggEC strains hybridised with the *astA* probe.

The role of EAggEC strains as a cause of diarrhoea in children has been a matter of controversy. They have been associated with diarrhoea in India and Mexico [13, 14], but, in contrast, they were found in similar percentages in both cases and controls from Brazil, Chile, Thailand and Bangladesh [11, 12, 15, 16]. From the present study, it seems that EAggEC strains are not associated with diarrhoea in children in Spain. The reason for these different results is not clear.

The in-vitro susceptibility of EAST-1-producing *E. coli* to several antimicrobial agents was investigated. The antimicrobial agents were distributed in three groups according to their activity. Group 1 comprised antimicrobial agents with good activity against EAST-1-producing *E. coli* (range of resistant strains from 0–10%), including cephalothin, cefuroxime, ceftazidime, cefoxitin, cefpirome, piperacillin, piperacillin plus tazobactam, imipenem, meropenem, aztreonam, trimethoprim-sulphamethoxazole, tobramycin, gentamicin, amikacin, norfloxacin and ciprofloxacin. Group 2 comprised antimicrobial agents with moderate activity (range of resistant strains 11–30%), including ticarcillin, tetracyclines, chloramphenicol and nitrofurantoin. Group 3 comprised antimicrobial agents with low activity (percentage of resistant strains > 30%) and contained only ampicillin.

In conclusion, the present study suggests that EAST-1-producing *E. coli* strains are associated with diarrhoeal diseases in Spanish children, whereas EAggEC strains are not. Moreover, EAST-1-producing *E. coli* strains showed a high susceptibility to all the antimicrobial agents tested except ampicillin.

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