ANTIMICROBIAL RESISTANCE

Susceptibility patterns of enteroaggregative *Escherichia coli* associated with traveller's diarrhoea: emergence of quinolone resistance

JORDI VILA, MARTHA VARGAS, JOAQUIM RUIZ, MATEU ESPASA, MARC PUJOL, MANEL CORACHÁN, M. TERESA JIMÉNEZ DE ANTA and JOAQUIM GASCÓN

Institut Clínic d'Infeccions i Immunologia, Institut d'Investigacions Biomèdiques August Pí i Sunyer (IDIBAPS), Hospital Clínic, University of Barcelona, School of Medicine, Villarroel 170, 08036 Barcelona, Spain

Enteroaggregative *Escherichia coli* (EAggEC) isolates were identified as a cause of traveller's diarrhoea in 50 (9%) of 517 patients and their antimicrobial susceptibility was determined. Molecular epidemiological characterisation and investigation of the mechanisms of acquisition of quinolone resistance among nalidixic acid-resistant EAggEC strains was performed. Seventeen (34%) of 50 patients needed antimicrobial therapy, because of persistence of symptoms in nine cases and the severity of symptoms in eight cases. Ampicillin and tetracycline resistance was high, whereas chloramphenicol and co-trimoxazole showed moderate activity and amoxicillin plus clavulanic acid, nalidixic acid and ciprofloxacin showed very good activity. Resistance to nalidixic acid was demonstrated in three isolates, two from patients who had travelled to India. In all three strains the resistance was linked to mutations in the *gyrA* gene alone or in both *gyrA* and *parC* genes. Although ciprofloxacin shows excellent in-vitro activity and could be useful in the treatment of traveller's diarrhoea in patients travelling abroad, it may not be useful in patients who have journeyed to India or to Mexico.

Introduction

Enteroaggregative *Escherichia coli* (EAggEC) are a less than well-defined category of diarrhoeagenic *E. coli*, as their exact mechanism of pathogenicity is not known. However, they are an important cause of acute and persistent infant diarrhoea in less-developed countries [1–4] and of traveller's diarrhoea [5, 6]. Recently, they have been associated with diarrhoea in industrialised countries [7–9] and with diarrhoea in patients with human immunodeficiency virus infection [10]. EAggEC cause a characteristic adherence pattern to HEp-2 cells as they attach to one another and to the HEp-2 cells [11–13]; this is a plasmid-dependent characteristic [14]. Genes encoding different virulence factors such as bundle-forming fimbriae involved in the adherence and a heat-stable toxin (EAST) have been localised in this plasmid [14, 15]. EAST, initially reported in EAggEC [15, 16], has also recently been found in other types of enteropathogenic *E. coli* [17].

A specific PCR technique has been developed to detect EAggEC causing diarrhoea [18].

The majority of enteric infections causing diarrhoea do not require antimicrobial therapy. However, in some infections treatment with an antimicrobial agent shortens the duration of shedding in faeces. This could be the case in persistent traveller's diarrhoea caused by EAggEC. Knowledge of local antimicrobial resistance patterns is important in selecting therapy. Therefore, this study reports the in-vitro activities of several selected antimicrobial agents against isolates of EAggEC causing traveller's diarrhoea from patients who had travelled to different tropical countries, in contrast to other published studies which were restricted to certain geographical areas [19, 20]. The molecular basis of nalidixic acid resistance among nalidixic acid-resistant EAggEC isolates was also investigated.

Materials and methods

Bacteria

During the period 1994–1997, stool specimens from 520 patients with traveller's diarrhoea were analysed. All patients had diarrhoeal illness on arrival in Spain or

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*Corresponding author: Dr J. Vila (e-mail: vila@medicina.ub.es).

developed it within 2 days of their return. The samples were collected during the acute phase of the diarrhoea and were processed within 2 h of collection. The stool specimens were cultured for *E. coli* and other bacterial enteropathogens by conventional methods [21]. Single colony subcultures of all different lactose-fermenting colonial morphotypes growing on MacConkey agar were identified by conventional criteria. These colonies were tested by PCR to detect EAggEC as described elsewhere [22].

Susceptibility testing

The MICs for the clinical isolates were determined by E-test methods according to standard practice. E-test strips (AB-Biodisk, Sölna, Sweden) were placed radially on Mueller-Hinton Agar plates inoculated with a suspension of 0.5 MacFarland standard density. After overnight incubation at 35°C, MICs were read at the intersection of the zone of growth inhibition with the strip. *E. coli* ATCC 25922 was used as a reference strain for quality control.

Amplification of the quinolone-resistant determining regions (QRDR) of the *gyrA* and *parC* genes by PCR

PCR was performed as described elsewhere [23, 24]. The PCR reaction was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, CA, USA). Primers and free nucleotides were removed with a QiaQuick spin PCR purification kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions; the sample was processed directly for DNA sequencing with a TaqDyeDeoxyTerminator Cycle Sequencing kit (Amersham, Cleveland, OH, USA) and analysed in an automatic DNA sequencer (Applied Biosystems 377A).

Low-frequency restriction analysis of chromosomal DNA

Genomic DNA for low frequency restriction analysis and pulsed-field gel electrophoresis (PFGE) was prepared in agarose plugs and treated as described previously [25]. One DNA insert was incubated overnight with 50 units of *Xba* (Promega, Madison, WI, USA) according to the manufacturer's instructions. Resultant DNA fragments were separated in an agarose 1% w/v gel (BioRad, Richmond, CA, USA) that was prepared and run in Tris-borate-EDTA buffer in a contour-clamped homogeneous field apparatus (CHEF-DR2, BioRad). The conditions of electrophoresis were 200 V for 20 h with pulse times of 1–50 s. Thereafter, the gel was stained with ethidium bromide and photographed.

Results

Isolation of EAggEC

EAggEC were isolated from 50 (9.6%) of 520 patients with traveller's diarrhoea. In four patients, another enteropathogen was isolated as well as EAggEC; these were *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, *Entamoeba histolytica* and *Cyclospora cayetanensis*. Seventeen (34%) of 50 patients needed antimicrobial therapy, because of the persistence of symptoms in nine cases and the severity of symptoms in eight cases. All the treated patients received ciprofloxacin and recovery was observed in all. The regions visited by the patients with traveller's diarrhoea caused by EAggEC are shown in Table 1. EAggEC was isolated from patients with traveller's diarrhoea who had travelled to different geographical areas around the world, with frequencies ranging from 2% to 20%. West Africa, the Indian subcontinent and south-east Asia were the most prevalent areas with 20%, 14% and 15%, respectively.

Antimicrobial susceptibility testing

The in-vitro susceptibility of the EAggEC isolates to seven antimicrobial agents is shown in Table 2. Ciprofloxacin, nalidixic acid and amoxicillin plus clavulanic acid showed the best activity against EAggEC; all gave MIC₅₀ and MIC₉₀ values below the breakpoint, whereas chloramphenicol and co-trimoxazole showed moderate activity with MIC₅₀ below the breakpoint and MIC₉₀ above the breakpoint. Ampicillin and tetracycline, both with a MIC₅₀ and MIC₉₀ values >256 mg/L, had the lowest levels of activity.

Characterisation of quinolone-resistant isolates of EAggEC

Three EAggEC isolates were resistant to nalidixic acid, two of which were isolated from patients travelling to India, representing 12.5% of the EAggEC isolates from patients who visited that country. The third nalidixic acid-resistant EAggEC isolate was from a traveller to

Table 1. Geographic distribution of EAggEC isolates from patients with traveller's diarrhoea

Geographical area	Number of patients	Number (%) of EAggEC isolates
East Africa	35	1 (3)
West Africa	61	12 (20)
Central Africa	12	1 (8)
Mahgreb	47	1 (2)
Middle East	21	2 (9)
South-east Asia	21	3 (14)
Indian subcontinent	106	16* (15)
Central America	160	10* (6)
South America	54	4 (7)
Total	517	50 (9)

*Of three nalidixic acid-resistant EAggEC isolates, two were from India and one from Central America.

Table 2. In-vitro susceptibility of 50 EAggEC isolates to seven antimicrobial agents

Antimicrobial agent	MIC Range	MIC50	MIC90	Percent of isolates resistant
Ampicillin	1–>256	>256	>256	52
Amoxicillin/clavulanic acid	0.75–12	4	6	0
Chloramphenicol	1–>256	4	>256	28
Tetracycline	1.5–>256	>256	>256	64
Trimethoprim-sulphamethoxazole	0.75–>32	0.75	>32	48
Nalidixic acid	1–>256	4	8	6
Ciprofloxacin	0.003–32	0.012	0.023	2

Central America. The two isolates (244DV and 164DV) from travellers to India had MICs to ciprofloxacin of 0.064 and >32 mg/L, respectively, whereas the MICs of nalidixic acid were >256 mg/L in both isolates. Isolate 232DV from a traveller to Central America had an MIC to ciprofloxacin of 0.25 mg/L and to nalidixic acid of >256 mg/L. PCR amplification of the *gyrA* and *parC* regions followed by DNA sequencing was used to investigate the implications of mutations in the QRDR of the *gyrA* and *parC* genes as a mechanism of acquisition of resistance to quinolones. Isolates 232DV and 244DV showed a mutation at amino acid codon Ser-83 which produced a substitution to Leu and Ala, respectively (Table 3). Meanwhile, isolate 164DV showed two mutations in the QRDR of the *gyrA* gene and two in the QRDR of the *parC* gene, which generated substitutions of Ser-83 to Leu and Asp-87 to Asn in the GyrA protein and Ser-80 to Ile and Glu-84 to Gly in the ParC protein (Table 3). The two nalidixic acid-resistant EAggEC isolates from travellers to India were investigated by PFGE to determine their clonal relationship. The DNA patterns obtained by PFGE clearly showed that the two strains were not epidemiologically related (Fig. 1).

Discussion

Recently, in a case-control study to elucidate the role of EAggEC as a cause of traveller's diarrhoea, Gascón *et al.* [5] found that EAggEC strains were isolated significantly more often from cases than from controls. Cohen *et al.* [26] found EAggEC in 12–13% of the

Fig. 1. PFGE of the three nalidixic acid-resistant EAggEC isolates from cases of traveller's diarrhoea. Lane M, DNA molecular mass markers; 1, isolate 164DV (travel to India); 2, 232 DV (travel to Central America); 3, 244 DV (travel to India).**Table 3.** Mutations in the *gyrA* and *parC* genes of quinolone-resistant clinical isolates of EAggEC

Isolates	MIC (mg/L)		Amino acid change in			
	Cip	Nal	GyrA		ParC	
Wild-type	...	...	Ser-83	Asp-87	Ser-80	Glu-84
244DV	0.064	48	Ala	...	...	...
232DV	0.25	256	Leu	...	...	...
164DV	>32	>256	Leu	Asn	Ile	Gly

Cip, ciprofloxacin; Nal, nalidixic acid.

patients who have travelled to Central or South America, the Caribbean or Mexico. In contrast, the results of the present study showed that the prevalence of EAggEC causing traveller's diarrhoea in travellers to Central and South America was 6% and 7% respectively. In the present study, the most prevalent area was West Africa (20%), and although no studies have been performed before in travellers to this geographical area, persistent diarrhoea caused by multidrug-resistant EAggEC has recently been reported in Kenyan [19] and Tanzanian [27] children. Another area of high prevalence found in the present study was the Indian subcontinent (15%). The importance of EAggEC as a cause of diarrhoea in children in this geographical area has been shown in several studies [1, 28, 29].

Knowledge of local antimicrobial resistance patterns is important in selecting therapy, as susceptibilities for an isolate will generally not be known until 72 h after the sample is processed. The present study did not find major differences in antimicrobial resistance among EAggEC from different geographical areas. Overall, they show a high to moderate level of resistance to ampicillin, tetracycline, co-trimoxazole and chloramphenicol. The emergence of resistance to these antimicrobial agents in other enteropathogens causing traveller's diarrhoea has also been described [28, 30–33]. Amoxicillin plus clavulanic acid, nalidixic acid and ciprofloxacin showed the best activity against EAggEC. Yamamoto *et al.* [20] investigated the in-vitro susceptibilities of EAggEC strains to various antimicrobial agents, demonstrating the appearance of marked drug resistance. However, this study was restricted to certain geographical areas – of 63 EAggEC strains analysed, 10 strains were from Mexico, Chile and Peru and 53 strains were from Thailand. The percentage of strains resistant to co-trimoxazole was 34.9%, a little lower than that found in the present study (48%). They suggested the potential usefulness of some antimicrobial agents, such as the fluoroquinolones, as no quinolone-resistant strains were found in their study. However, in the present study, three EAggEC isolates were resistant to nalidixic acid and one of them was also highly resistant to ciprofloxacin. Of these three isolates, two were from travellers to India and the third from a traveller to Central America. Analysis of the two nalidixic acid-resistant isolates from India by PFGE demonstrated that they were not epidemiologically related.

The nalidixic acid resistance in these three EAggEC isolates was due to a mutation in amino acid codon Ser-83 of the *gyrA* gene in the isolates resistant to nalidixic acid but susceptible to ciprofloxacin and to four mutations, two in the *gyrA* gene and two in the *parC* gene, in the isolate that was resistant to nalidixic acid and ciprofloxacin. This confirms previous findings that high-level resistance to ciprofloxacin in *E. coli* is related to the presence of concomitant mutations in the *gyrA* and *parC* genes [23, 24].

The fluoroquinolones have received considerable attention and have been shown to be highly effective in reducing the duration of traveller's diarrhoea [34–37]. Recently, Glandt *et al.* [6] showed a significant reduction in the duration of post-treatment diarrhoea and a non-significant reduction in the mean number of unformed stools passed during the 72 h after enrolment in the ciprofloxacin-treated group as compared with that of the placebo group.

In summary, oral antimicrobial agents commonly used to treat bacterial diarrhoea, particularly co-trimoxazole, have poor in-vitro activity against EAggEC. Despite the excellent in-vitro activity of ciprofloxacin, demonstrating its possible usefulness in the treatment of diarrhoea in patients travelling abroad, the possibility of resistance to this antimicrobial agent should be considered in patients with traveller's diarrhoea who have journeyed to India or to Mexico.

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