Emerging pathogen Arcobacter spp. in acute gastroenteritis: molecular identification, antibiotic susceptibilities and genotyping of the isolated arcobacters

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The aims of this study were as follows: (i) to isolate Arcobacter spp. from the stool samples of patients with gastroenteritis; (ii) to identify them with molecular methods; (iii) to genotype them using enterobacterial repetitive intergenic consensus (ERIC)-PCR; and (iv) to determine their antibiotic susceptibilities. For the study, a total of 3287 diarrhoeal stool samples submitted to the Microbiology Laboratory of the Kayseri Training and Research Hospital, Kayseri, Turkey, between 2010 and 2011 were analysed. Campylobacter blood-free selective medium supplemented with cefoperazone, amphotericin B and teicoplanin was used for isolation. Medium inoculated with stool samples was incubated microaerobically at 37 °C for 72–96 h. Phenotypic tests, a genus-specific PCR and a multiplex PCR were used to identify the arcobacters, whilst ERIC-PCR was used for genotyping and the antibiotic susceptibilities of the isolates were detected by E-test. Arcobacter spp. were isolated from nine of the 3287 samples. These nine isolates were identified as Arcobacter butzleri and all showed different ERIC-PCR profiles. All nine isolates were resistant to ampicillin and susceptible to gentamicin, tetracycline, erythromycin and ciprofloxacin. As far as is known, this is the first study in which A. butzleri has been isolated from human acute gastrointestinal infections in Turkey. According to these results, it is recommended that, when investigating the aetiology of infections of the digestive system in humans, Arcobacter spp. be considered for inclusion. The results of this study should contribute to our knowledge related to A. butzleri infections in humans.

INTRODUCTION

The genus Arcobacter is a member of the family Campylobacteraceae. Currently, the genus Arcobacter has a total of 15 recognized species: Arcobacter butzleri, Arcobacter cryaerophilus, Arcobacter skirrowii, Arcobacter nitrofigilis, Arcobacter cibarius, Arcobacter halophilus, Arcobacter mytili, Arcobacter thereius, Arcobacter marinus, Arcobacter trophiarum, Arcobacter defluvii, Arcobacter molluscorum, Arcobacter ellisi, Arcobacter bivalviorum and Arcobacter venerupis (Collado & Figueras, 2011; Figueras et al., 2011; Figueras et al., 2011; Levcian et al., 2012). Three species, namely A. butzleri, A. cryaerophilus and A. skirrowii, have been associated with gastrointestinal infections (Burnens et al., 1992; Collado & Figueras, 2011; Jiang et al., 2010; Vandamme et al., 1992a) and extra-intestinal invasive diseases (Lau et al., 2002; On et al., 1995; Yan et al., 2000). In addition, asymptomatic arcobacter carriage in type 2 diabetic patients has been reported (Fera et al., 2010). Because of its isolation from many human disease cases, A. butzleri is considered to be the most important species of the genus. Some studies have reported the isolation and molecular analyses of arcobacters from miscellaneous sources in Turkey (Atabay et al., 2003, 2008; Aydin et al., 2007; Ertas et al., 2010). However, in human medicine, no studies have been conducted on arcobacters, except for one case report (Kayman et al., 2010). The current study was undertaken to determine the prevalence of Arcobacter spp. in acute gastroenteritis in Kayseri, Turkey. In addition, the isolates were identified at the species

Abbreviations: CAT, cefoperazone, amphotericin B and teicoplanin; CLSI, Clinical and Laboratory Standards Institute; ERIC, enterobacterial repetitive intergenic consensus.
level using a multiplex PCR, genotyped by enterobacterial repetitive intergenic consensus (ERIC)-PCR and tested against various antibacterials by E-test.

**METHODS**

In this study, a total of 3287 stool samples of patients with diarrhoea that were sent to the Kayseri Training and Research Hospital Microbiology Laboratory, Kayseri, Turkey, from different departments, between 1 March 2010 and 1 March 2011 were used.

**Isolation and identification of Arcobacter spp.** Campylobacter blood-free selective medium (modified CCDA-Preston; Oxoid) supplemented with cefoperazone, amphotericin B and teicoplanin (CAT; Oxoid) was used to isolate Arcobacter spp. strains. The stool specimen was plated onto this medium by direct streaking, and the inoculated plates were incubated at 37 °C under microaerobic conditions for 72–96 h. In general, arcobacters, like campylobacters, generate grey colonies with a metallic sheen on CAT-supplemented modified CCDA-Preston agar. Colony morphology, Gram staining, oxidase and catalase tests, evaluation of motility by phase-contrast microscopy, growth under aerobic conditions and a growth test at 30 °C were used for phenotypic identification of Arcobacter spp. (Aydin et al., 2007; Collado & Figueras, 2011). Colonies that proved to be Gram-negative, slightly curved rods, oxidase positive, catalase positive, motile and able to grow under both aerobic conditions and at 30 °C were identified as Arcobacter spp.

**DNA extraction.** DNA extraction was carried out by the method reported by Houf et al. (2000). Each of the isolates was streaked onto blood agar and incubated under microaerobic conditions for 24–48 h. Growing colonies of arcobacter were picked and homogenized in 1 ml distilled water. After centrifugation at 13 000 r.p.m. in a Genofuge (Techne) for 5 min, the pelleted bacteria were resuspended in 500 ml sterile distilled water. Extraction of target DNA was achieved by boiling the suspension for 10 min, followed by centrifugation at 13 000 r.p.m. as above for 10 min. Before the supernatant was used as a target DNA, the DNA concentration was adjusted to 25 ng μl⁻¹ following measurement of absorbance at 260 nm in a spectrophotometer (Genova).

**Identification of Arcobacter spp. isolates at the genus level.** In order to confirm the isolates identified as Arcobacter spp. by phenotypic tests, a genus-specific PCR was performed, as reported by Harmon & Wesley (1996). For this procedure, the primer pair ARCOI/ARCOII targeting a section of the 16S rRNA gene was used. The samples were subjected to an initial denaturation step at 94 °C for 1 min, followed by 35 amplification cycles of 1 min at 94 °C (denaturation), 1 min at 56 °C (primer annealing) and 1 min at 72 °C (primer extension) carried out in a Touchgene Gradient thermal cycler. A final extension step consisted of 7 min at 72 °C. The amplified products were resolved in 0.6 % (w/v) Tris-acetate-EDTA (TAE) agarose gel and the band patterns were analysed in a gel documentation system (Vilber Lourmant). For the genus Arcobacter, a band of 1223 bp indicated a positive result. Sterile distilled water in place of DNA served as a negative control.

**Identification of Arcobacter spp. isolates at the species level.** For identification of Arcobacter spp. isolates at the species level, the multiplex PCR method reported by Houf et al. (2000) was carried out. In the test, primers ARCO, BUTZ, CRY1, CRY2 and SKIR were used. Amplification of DNA consisted of an initial denaturation at 94 °C for 2 min, followed by 32 cycles of 94 °C for 45 s, 61 °C for 45 s and 72 °C for 30 s carried out in a Touchgene Gradient thermal cycler. The amplified products were resolved in 1.5 % (w/v) TAE agarose gel at 100 V for 40 min (EC340 Maxicell, Thermo). The resulting bands were visualized in a gel documentation system (Vilber Lourmant). Predicted band sizes were 401 bp for A. butzleri, 257 bp for A. cryaerophilus and 641 bp for A. skirrowii. The reference strain A. butzleri LMG 10828 was included as a positive control. In addition, previously identified isolates of A. cryaerophilus and A. skirrowii were used as controls in the assay.

**ERIC-PCR and electrophoresis.** ERIC-PCR and electrophoresis were carried out using the method of Houf et al. (2002). Briefly, the total 50 μl volume of prepared PCR mixture consisted of 5 μl 10 × PCR buffer, 4 mM MgCl₂, 5 U Taq polymerase, dNTP mix at a final concentration of 0.2 mM (all from Fermentas), 25 pmol primers 1R (5’-ATGTAAGCTCCTGGGGATTCAC-3’) and 2 (5’-AAGTAGTGACTGGGGGTGAGCG-3’) (Houf et al., 2002) and 1 μl target DNA. DNA amplification consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 25 °C for 1 min and 72 °C for 2 min. The amplified products were resolved by electrophoresis in a 2 % (w/v) TAE agarose gel at 100 V for 1 h. The gel was analysed and evaluated by visual inspection under UV light for distinct DNA profiles to detect polymorphisms among the isolates (Aydin et al., 2007; Houf et al., 2002).

**Antibiotic susceptibility test.** Antibiotic susceptibility testing of the A. butzleri isolates was carried out using the E-test method. Mueller–Hinton agar (Oxoid) supplemented with 5 % sheep blood was used in the test. The antibiotics tested were ampicillin, gentamicin, erythromycin, tetracycline (all from Oxoid), nalidixic acid and ciprofloxacin (both from bioMérieux). *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Campylobacter jejuni* ATCC 33560 and A. butzleri LMG 10828 were used as control strains. Because there is no standard procedure for the antibacterial susceptibility testing of arcobacters, we used Clinical and Laboratory Standards Institute (CLSI) guidelines M45-A (for campylobacters) for erythromycin.

**Fig. 1.** Identification of Arcobacter isolates at the species level by multiplex PCR. Lanes: M, size marker (GeneRuler 100 bp DNA ladder; Fermentas); 1–9, A. butzleri isolates; N, negative control (sterile distilled water); 10, A. butzleri LMG 10828; 11, A. skirrowii (positive control); 12, A. cryaerophilus (positive control)
tetracycline and ciprofloxacin (CLSI, 2006), and CLSI M100–S20 (for Enterobacteriaceae) for ampicillin, gentamicin and nalidixic acid (CLSI, 2010).

RESULTS

Isolation and identification

Nine (0.3 %) of the 3287 stool samples tested were found to be positive for Arcobacter spp. by phenotypic tests. All isolates showed a 1223 bp band in the genus-specific PCR analysis and hence were identified as Arcobacter spp. All showed a 401 bp band in the multiplex PCR and were thus identified as A. butzleri (Fig. 1). In addition, these nine samples were found to be negative for other enteric pathogens such as Salmonella spp., Shigella spp., Campylobacter spp., rotavirus, adenovirus, parasites and parasite eggs.

Patients

The nine patients from whom A. butzleri was isolated comprised four children (three boys, one girl) and five adults (three females, two males). The age range of the paediatric patients was 4–16 years and the adults were aged between 23 and 66 years. The ages and genders of the patients are shown in Table 1. The common symptoms of all patients were nausea, abdominal pain with cramps, and acute diarrhoea. Acute diarrhoea was described as at least three unformed or liquid stools per day. In three of the adult patients, the diarrhoea was watery, whilst the other patients had unformed stools. Diarrhoea continued for 1–3 days. In two of the paediatric patients, vomiting was accompanied by diarrhoea. One paediatric patient had a fever of 38.5 °C. By microscopic examination, leukocytes and mucus were observed in the stool sample of one paediatric patient. In the study, four, one, one, one and two cases were detected in January, March, August, September and November, respectively. The clinical features of the nine patients are given in Table 2.

ERIC-PCR

The ERIC-PCR band patterns in 2 % agarose gel are shown in Fig. 2. In general, bands of between 150 and 950 bp were obtained, and the ERIC-PCR analysis revealed nine different profiles.

Antibiotic susceptibility test

The results of the antibiotic susceptibility tests are shown in Table 3. All nine isolates were found to be resistant to ampicillin and susceptible to erythromycin, gentamicin, tetracycline and ciprofloxacin. Two isolates were resistant to nalidixic acid, whilst seven isolates were found to be susceptible.

DISCUSSION

In this study, the prevalence of Arcobacter spp. in gastroenteritis was evaluated, and identification, and antibiotic susceptibility and genotyping of the isolated strains were carried out. These bacteria, which were formerly classed in the genus Campylobacter (Kiehlbauch et al., 1991; Vandamme et al., 1991) but, as a result of detailed taxonomic studies, were later assigned to the genus Arcobacter (Vandamme et al., 1991, 1992b), are included among foodborne and waterborne enteric pathogens. It has been reported from both sporadic cases and certain periodic studies that arcobacters are implicated in the aetiology of gastroenteritis (Lerner et al., 1994; Vandamme et al., 1991) but, as a result of detailed taxonomic studies, were later assigned to the genus Arcobacter (Vandamme et al., 1991, 1992b), are included among foodborne and waterborne enteric pathogens. It has been reported from both sporadic cases and certain periodic studies that arcobacters are implicated in the aetiology of gastroenteritis (Lerner et al., 1994; Vandamme et al., 1992a; Vandenberg et al., 2004). Arcobacters can cause different clinical manifestations (Vandenberg et al., 2004). An outbreak occurred in a school as reported by Vandamme et al. (1992a), two diarrhoea cases were reported by Lerner et al. (1994) and two chronic diarrhoea cases were reported by Fernandez et al. (2004). In addition, arcobacters were isolated from tourist diarrhoea cases of USA and European tourists who travelled to Mexico, Guatemala and India at a rate of 8 %, as

Table 1. Age and gender distribution of the patients from whom A. butzleri was isolated

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>1 2 3 4 5 6 7 8 9</td>
</tr>
<tr>
<td>Gender</td>
<td>F M M M M F M F</td>
</tr>
</tbody>
</table>

Table 2. Clinical features of the patients with A. butzleri infection

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>No. patients/total</th>
</tr>
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<tbody>
<tr>
<td>Abdominal pain</td>
<td>9/9</td>
</tr>
<tr>
<td>Nausea</td>
<td>9/9</td>
</tr>
<tr>
<td>Vomiting</td>
<td>2/9</td>
</tr>
<tr>
<td>Fever</td>
<td>1/9</td>
</tr>
<tr>
<td>Watery diarrhoea</td>
<td>3/9</td>
</tr>
<tr>
<td>Unformed stool</td>
<td>6/9</td>
</tr>
<tr>
<td>Mucus in stool</td>
<td>1/9</td>
</tr>
<tr>
<td>Erythrocytes in stool</td>
<td>0/9</td>
</tr>
<tr>
<td>Leukocytes in stool</td>
<td>1/9</td>
</tr>
</tbody>
</table>
reported by Jiang et al. (2010). Vandenberg et al. (2004) examined 67,599 stool samples between 1995 and 2002 and found 77 (0.1%) of them to be positive for Arcobacter spp. In another study conducted by Patyal et al. (2011), over a 1-year period, arcobacters were isolated from human diarrhoeal stool samples at a rate of 2.67% using a culture method, whilst 4% of samples were positive using PCR screening after enrichment of specimens. Mandisodza et al. (2012) recently reported that Arcobacter spp. were isolated from human diarrhoeal faeces at a rate of 0.9% (12/1380) in New Zealand. In the current study, A. butzleri was isolated from nine (0.3%) of 3287 stool samples examined. All the patients had acute gastroenteritis, and the clinical and laboratory findings (Table 2) were similar to the results reported by Vandenberg et al. (2004), although the difference in isolation rates may have been due to the different isolation media used. Despite the use of different media by various authors (Hsueh et al., 1997; Merga et al., 2011; Patyal et al., 2011; Samie et al., 2007; Vandenberg et al., 2004; Wybo et al., 2004), standard isolation procedures for arcobacters have not been established.

In recent years, ERIC-PCR has been used to determine the molecular epidemiology of arcobacters isolated from a variety of environments such as poultry (Atabay et al., 2002; Houf et al., 2002), cattle (van Driessche et al., 2005) and food (Aydin et al., 2007), and it has been determined that this micro-organism has a large number of subtypes. In addition, Houf & Stephan (2007) typed seven isolates as A. cryaerophilus using ERIC-PCR and found that the seven isolates had seven different genotypes. Houf et al. (2004) reported that 30 A. butzleri and eight A. cryaerophilus isolates from clinical cases had 38 different genotypes by ERIC-PCR. The nine A. butzleri isolates from patients with gastroenteritis in our study had nine different profiles by ERIC-PCR. This emerging high heterogeneity is compatible with the ERIC-PCR findings of Houf & Stephan (2007) and Houf et al. (2004). Another study found that all arcobacters isolated from human diarrhoeal cases had different pulsotypes by PFGE typing (Mandisodza et al., 2012). The presence of a large degree of heterogeneity among the Arcobacter isolates observed in our study, as well as in earlier studies (Houf & Stephan, 2007; Houf et al., 2004), suggests that there are multiple sources of Arcobacter contamination in the environment.

Although arcobacters have been isolated from human infections since 1991, standardized procedures to determine antibiotic susceptibility patterns have not been established. However, several studies of antibiotic susceptibility from clinical cases have been reported (Houf et al., 2004; Mandisodza et al., 2012; Vandenberg et al., 2006). Houf et al. (2004) reported the antibiotic susceptibilities of 30 A. butzleri isolates from patients with acute or chronic diarrhoea carried out by the agar dilution method and found that all isolates were susceptible to ciprofloxacin and gentamicin, whilst they found different MIC values for erythromycin and nalidixic acid. In another study performed by Vandenberg et al. (2006), the antibiotic susceptibilities of clinical isolates were assessed by E-test. Whilst all of the 61 A. butzleri isolates were found to be susceptible to gentamicin and tetracycline, two isolates were resistant to ciprofloxacin and 11 of the isolates were resistant to nalidixic acid. They also found that 78.7% of the isolates were susceptible to ampicillin and erythromycin. Although in our study all isolates showed a resistance

Table 3. MIC values of the A. butzleri strains

| Antimicrobial | MIC range | S | I | R | 0.03 | 0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 |
|--------------|-----------|---|---|---|------|------|------|------|-----|----|---|---|---|---|----|----|----|----|----|
| Ampicillin   | $\leq 8$  | 16 | $\gg 32$ | 1 | 1 | 7 |
| Gentamicin   | $\leq 4$  | 8  | $\gg 16$ | 3 | 5 | 1 |
| Erythromycin | $\leq 8$  | 16 | $\gg 32$ | 2 | 4 | 2 | 1 |
| Ciprofloxacin| $\leq 1$  | 2  | $\gg 4$ | 3 | 5 | 1 |
| Tetracycline | $\leq 4$  | 8  | $\gg 16$ | 1 | 4 | 4 |
| Nalidixic acid| $\leq 16$ | $\gg 32$ | 1 | 2 | 4 | 1 | 1 |
rate of 100% to ampicillin, they were fully susceptible to erythromycin, gentamicin, ciprofloxacin and tetracycline. Similar to our findings, in a recent study conducted by Mandisodza et al. (2012), 12 A. butzleri isolates recovered from diarrhoeic patients were found to be susceptible to ciprofloxacin and erythromycin at rates of 100 and 92%, respectively. Moreover, the resistance rate of arcobacters to ampicillin was 50%. The resistance rate of erythromycin reported by Houf et al. (2004) did not correlate with our results. This difference may be due to the method used in the antibiotic susceptibility test. Ciprofloxacin and erythromycin are the antibiotics of choice in the treatment of Campylobacter infections. However, although in 1994 among 119 Campylobacter spp. isolates only one was resistant to quinolone as reported by Akan et al. (1994), in 2010 quinolone resistance was found at a rate of 64.3% in a study conducted by Guneý & Basustaoglu (2010). Quinolone use in livestock to prevent infections, encourage rapid growth and increase meat production is thought to have contributed to the development of this resistance. Despite the taxonomic proximity of arcobacters to campylobacters, the quinolone resistance of arcobacters was evaluated at a lower level in both our study and other studies (Houf et al., 2004; Mandisodza et al., 2012; Vandenbergh et al., 2006). This could be due to the few studies conducted on arcobacters so far.

To our knowledge, this is the first study to be carried out in Turkey on the isolation of arcobacters from gastroenteritis, the identification of isolates with molecular tests, genotyping with ERIC-PCR and determining the antibacterial susceptibility of the isolates. According to these results, it is recommended that, when investigating the etiology of gastrointestinal infections in humans, Arcobacter spp. be considered for inclusion. We believe that our study will contribute to the knowledge related to A. butzleri infections in humans.

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