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Isolation and identification of *Arcobacter* species from environmental and drinking water samples

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Abstract Water plays an important role in the transmission of *Arcobacter* spp. to animals and humans. The aim of this study was to isolate and characterize *Arcobacter* spp. from 115 different water samples (66 sewage, 25 rivers, 16 spring water, and 8 drinking water) in Izmir, Turkey. In total, 41 samples (35.7 %) were found positive for *Arcobacter* spp. by the genus-specific PCR. *Arcobacter butzleri* was detected in 39 out of 115 samples (33.9 %) including 24 sewage, 13 rivers, and 2 spring water. The remaining *Arcobacter* spp. ($n=2$) isolates could not be identified by m-PCR and 16S rRNA gene sequencing. Based on the phenotypic characterization, most of the *Arcobacter* species (87.8 %) indicated weak catalase activity. In addition, there were differences in phenotypic patterns among isolated species during growth at 37 °C under microaerobic and aerobic conditions, in the presence of 2 % (39/41) and 3.5 % (32/41) NaCl and 0.04 % TTC (39/41) and on MacConkey agar (38/41). The results of this study indicated that environmental water samples are common sources for *Arcobacter* spp. Therefore, effective control measures should be taken to protect human health.

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Introduction

The members of the genus *Arcobacter* are Gram-negative, fastidious, microaerophilic, non-spore-forming, usually motile, spiral-shaped organisms in the family of *Campylobacteraceae*. They differ from closely related campylobacters for their ability to grow microaerobically or aerobically at lower temperatures ranging from 15 to 37 °C (Vandamme and De Ley 1991). Although it is not a member of normal flora of human intestine, the consumption of contaminated foods of animal origin and water can result in human infections (Shah et al. 2011). *Arcobacter* spp. have been isolated from various sources such as water, foods of animal origin, clinical samples, food-processing equipment, and water distribution pipes (Phillips 2001). The genus *Arcobacter* currently includes 21 species (Giacometti et al. 2015). Among these species, *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* are the most important species due to their common association with human diseases (Ferreira et al. 2015). Clinical symptoms of *Arcobacter* infection are abdominal cramp and watery diarrhea (Collado and Figueras 2011).

Arcobacter prevalence has been detected in many countries. Understanding the potential risks related with this foodborne and waterborne pathogen is necessary (Hsu and Lee 2015). Since contaminated water is one of the possible sources of infection in animals and humans (Ho et al. 2006), to determine the prevalence of *Arcobacter* in water is useful to better understand the transmission process of these infectious agents, ecological characteristics, and zoonotic potential risks associated with water (Çelik and Ünver 2015). Wastewater (González et al. 2007; Collado et al. 2008; Merga et al. 2014), seawater (Collado et al. 2008; Fera et al. 2010; Ghane 2014), lakes and rivers (Collado et al. 2008; 2010), drinking water (Ertas et al. 2010; Jalava et al. 2014), groundwater (Fong et al. 2007), and recreational water (Lee et al.

2012) were found positive for the presence of *Arcobacter* spp. Recently, there is limited information in terms of the prevalence of *Arcobacter* spp. in water from Turkey (Ertas et al. 2010; Çelik and Ünver 2015). Therefore, the present study was aimed to isolate and characterize *Arcobacter* spp. from different water sources using phenotypic and molecular methods in Izmir, Turkey.

Material and methods

Bacterial strains

Reference strains of *A. butzleri* (LMG 10828), *A. cryaerophilus* 1A (LMG 9904) and 1B (LMG 10229), and *A. skirrowii* (LMG 6621) were kindly provided by Maria José Figueras from University of Rovira I Virgili, Spain.

Sample collection

A total of 115 samples including sewage ($n = 66$), river ($n = 25$), spring water ($n = 16$), and drinking water ($n = 8$) were collected from 23 different sampling sites in Izmir and surrounding areas during a period of 7 weeks (February to April 2011). They were placed in separate sterile plastic bottles, transferred to the laboratory, and processed immediately.

Isolation

Briefly, 200 mL of sample was centrifuged at 3500*g* for 15 min. Then, the supernatant was discarded. The resulting pellet was suspended in 20 mL *Arcobacter* Broth (Oxoid CM965, UK) with CAT supplement (Cefoperazone, Teicoplanin, Amphotericin B, Oxoid SR174, UK). The broths were incubated under microaerophilic conditions using an automated anaerobic system (Anoxomat, Mart Microbiology, The Netherlands) at 30 °C for 3 to 4 days. After enrichment, membrane filtration technique was applied using 0.45-μm pore size nitrocellulose membrane filters (Millipore HAWG047S1, USA) as described previously (Atabay and Corry 1997). Briefly, 100 μL of the enriched culture was pipetted onto the surface of membrane that had been placed onto the surface of blood agar base (Oxoid CM0055, UK) supplemented with 5 % (*v*/*v*) sheep blood. After incubation at room temperature for 30 min, the filters were carefully removed and discarded. Then, the filtrates were evenly distributed over the agar surface with a sterile spreader. Finally, the plates were incubated at 30 °C for 48–72 h under microaerobic conditions. Two to three suspected colonies (pin-pointed, translucent, and watery colonies) (Shah et al. 2012a) were selected from each plate and then subcultured by streaking on modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid CM739, UK). Pure cultures were tested for Gram staining, oxidase test (Merck 1.13300, Germany), and motility under phase contrast microscope. Gram-negative, spiral-shaped, motile, and oxidase-positive isolates were stored in 20 % (*v*/*v*) nutrient broth No. 2 (Oxoid CM0067, UK)-glycerol (AppliChem A1123, Germany) at −80 °C.

Phenotypic characterization

The isolates were characterized phenotypically using methods as previously described (Atabay and Corry 1997; Atabay et al. 2008). These tests included catalase and H_2S production, indoxyl-acetate hydrolysis (Fluka 04739, UK), growth at different temperatures under aerobic and microaerobic conditions, and growth in the presence of NaCl (2 and 3.5 %, *w*/*v*, AppliChem A1149, Germany) and 0.04 % TTC (Oxoid SR0229, UK) and on MacConkey agar (Oxoid CM0007, UK).

Genomic DNA isolation

DNA was extracted using a commercial genomic DNA isolation kit (PureLink® Kit, Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The concentration of each DNA was determined spectrophotometrically at 260 and 280 nm (NanoDrop 8000-Thermo Fisher Scientific, USA), adjusted to 20 ng/μL and stored at −20 °C.

Genus-specific PCR

For the genus-specific PCR, the primer combinations ARCOI-ARCOII targeting a section of the 16S ribosomal RNA (rRNA) genes were used (Harmon and Wesley 1996). The PCR reactions were performed in a 25-μL reaction mixture containing 2 μL template DNA, 2.5 μL of 10 × PCR buffer, 3 mmol/L $MgCl_2$, 10 μmol/L of each of the primers, 0.2 mmol/L each of the four dNTPs (Fermentas, Thermo Fisher Scientific, USA), and 1.5 U *Taq* DNA polymerase (Fermentas, Thermo Fisher Scientific, USA). The thermal cycling conditions were initial denaturation at 94 °C for 4 min, followed by 29 cycles consisting of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and primer extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. PCR experiments were repeated twice for each strain. The amplified products were electrophoresed on 1 % (*w*/*v*) Tris-acetate-EDTA (TAE, 0.04 mol/L Tris-acetate, 0.001 mol/L EDTA pH 8.0) agarose gel using 1 kb ladder (Fermentas, Thermo Fisher Scientific, USA). The band patterns were analyzed in a gel documentation system (Vilber Lourmat, France). The DNA of the reference strains were used as positive controls and sterile deionized water was used as a negative control in PCR experiments.

Multiplex PCR

The isolates identified as *Arcobacter* spp. were examined by m-PCR using the primers developed by Houf et al. (2000). The selected primers amplify a 257-bp fragment from *A. cryaerophilus*, 401 bp from *A. butzleri*, and 641 bp fragment from *A. skirrowii*. PCR reactions were performed in a 25-μL reaction mixture containing 2 μL template DNA, 2.5 μL of 10 × PCR buffer, 3 mmol/L $MgCl_2$, 10 μmol/L of each of the primers, 0.2 mmol/L each of the four dNTPs (Fermentas, Thermo Fisher Scientific, USA), and 1.5 U *Taq* DNA polymerase (Fermentas, Thermo Fisher Scientific, USA). The PCR reactions were performed in a thermal cycler Bio-Rad C-100 (Bio-Rad, USA) with the following amplification conditions: a denaturation step at 94 °C for 3 min; 34 amplification cycles: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min; and the final extension step at 72 °C for 7 min. The PCR products were analyzed on 1.25 % (*w*/*v*) TAE-agarose gel using 100 bp ladder (Fermentas, Thermo Fisher Scientific, USA). The band patterns were analyzed in the gel documentation system (Vilber Lourmat, France). DNA from reference strains and sterile deionized water were used as positive and negative controls, respectively.

16S ribosomal RNA gene sequencing

The 16S rRNA genes were amplified using the same primers and conditions used for the genus-specific PCR (Harmon and Wesley 1996). After purification, the amplicons were sequenced bidirectionally using the same primers by the ABI 3130 XL genetic analyzer (Applied Biosystems, Thermo Fisher Scientific, USA) with the Prism BigDye terminator cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific, USA). Partial 16S rRNA sequences were compared with the other 16S rRNA gene sequences in GenBank database to determine their sequence identities.

Results and discussion

Water is an important source for the transmission of *Arcobacter* species to both animals and humans (Hsu and Lee 2015). In the present study, the occurrence of *Arcobacter* spp. was investigated in 115 water samples (66 sewage, 25 rivers, 16 spring water, and 8 drinking water samples) collected from Izmir, Turkey.

Sixty-one isolates that were Gram-negative, oxidase-positive, and indicating typical cork-screw type motility were subjected to genus-specific PCR. Since PCR amplification of campylobacters from water samples is difficult due to the low levels in the environmental samples, a short pre-enrichment followed by a purification step of the isolated genomic DNA is necessary before PCR analysis (Giesendorf et al. 1993; Van Camp et al. 1993). Therefore, the genomic DNA was extracted from pure cultures obtained after pre-enrichment step. Based on the genus-specific PCR, 41 out of 61 isolates were identified as *Arcobacter* spp. yielding a 1223-bp fragment (Harmon and Wesley 1996). The overall prevalence of *Arcobacter* spp. in water was 35.7 %. Based on the type of sample, the prevalence was found as 52 % (13/25) in river water, 36.4 % (24/66) in sewage, and 25 % (4/16) in spring water samples. Similar to a previous report from Kars, Turkey (Çelik and Ünver 2015), all drinking water samples were negative for *Arcobacter* spp. in this study most probably due to effective chlorination disinfection used for water treatment.

In the related literature, the prevalence of *Arcobacter* spp. has been reported in different water samples such as 23 % in river and 100 % in canal water (Morita et al. 2004); 55.1 % in freshwater, seawater, and sewage samples (Collado et al. 2008); 3 % in drinking water and 1 % in spring water (Ertas et al. 2010); 75.2 % in recreational water (Lee et al. 2012); 11.11 % in chlorinated water (Shah et al. 2012a); 26.31 % in creek water and 18.36 % in stream water (Çelik and Ünver 2015); and 86.7 % in wastewater (Šilha et al. 2015). The variations in the presence of arcobacters in water may be due to use of different sample types, fecal contamination levels, seasonal variations, water temperature, and isolation method used. Fong et al. (2007) suggested that extreme rainfall may provide in the transport of *Arcobacter* from wastewater treatment plants to groundwater. In another study, Lee et al. (2012) showed that the presence in recreational water was higher in September and the levels indicated a negative correlation with water temperature. It is known that *Arcobacter* spp. may survive better at lower temperatures in water (Fera et al. 2010). Also, the presence of *Arcobacter* spp. in water increases markedly with high levels of fecal contamination (Collado et al. 2008). As mentioned before, the method used for isolation may be effective on the recovery rates but there is no standardized protocol available for the isolation (Shah et al. 2012b). In this study, membrane filtration technique after selective enrichment in *Arcobacter* Broth with CAT supplement was used to eliminate the incidence of background flora. The use of selective supplement may lead to lower recovery rates of *Arcobacter* spp. in environmental water samples. Since the cells in environmental samples are stressed or injured, it has been reported that the use of inhibitory compounds may inhibit the growth of environmental isolates resulting in reduced recovery rates of injured cells of *Campylobacter* strains (Diergaardt et al. 2004).

The m-PCR indicated that *A. butzleri* was the only one recovered from 33.9 % of the samples (Table 1). Previous studies have also showed that it is the most frequently associated species with water sources (Hsu and Lee 2015). In total, 21 samples including sewage ($n = 13$), river water ($n = 7$), and

Table 1 Distribution of *Arcobacter* spp. in water based on the sample type and method used for species identification

Source	No. of samples collected	No. of *Arcobacter* spp.	No. of *A. butzleri* identified by m − PCR	No. of *A. butzleri* identified by 16S rRNA sequencing	Unidentified
Sewage	66	24 (36.4 %)	13 (19.7 %)	11 (16.7 %)	–
River	25	13 (52 %)	7 (28 %)	6 (24 %)	–
Spring water	16	4 (25 %)	1 (6.25 %)	1 (6.25 %)	2 (12.5 %)
Drinking water	8	–	–	–	–
Total	115	41 (35.7 %)	21 (18.3 %)	18 (15.7 %)	2 (1.74 %)

spring water ($n = 1$) were positive for *A. butzleri* by m-PCR. A high genetic diversity existing within the genus and also between the species has been observed (Collado et al. 2010; Kayman et al. 2012). The remaining *Arcobacter* species ($n = 18$) that were not identified with m-PCR were subjected to sequencing of 1223 bp of fragment within the 16S rRNA gene. Based on sequencing, most of these isolates ($n = 18$) were assigned to *A. butzleri* with high level of similarities ranging from 96 to 99 %. These strains were obtained from sewage ($n = 11$), river water ($n = 6$), and spring water ($n = 1$). Two *Arcobacter* species from spring water could not be identified using m-PCR and sequencing (Table 1). The exact reasons for failure in identification of these isolates could be due to the lack of appropriate sequence of isolates and/or they could be a possible new strain/species.

The International Commission on Microbiological Specification for Foods (ICMSF 2002) has considered *A. butzleri* to be a significant hazard to human health. Interestingly, 63 % of *A. butzleri* infections in humans occur mainly through consumption or close contact with contaminated water (Shah et al. 2011). *A. butzleri* can easily attach to water distribution pipe surfaces (stainless steel, copper, and plastic) which causes regrowth in the water distribution systems. Therefore, it is a significant problem in drinking water and food-processing plants with respect to public health (Assanta et al. 2002). In fact, the overall recovery rates of *A. butzleri* were 52 % (13/25) in river water, followed by 36.4 % (24/66) in sewage water and 12.5 % (2/16) in spring water. In this study, sampling was done in the spring (February to April). These results were lower than those of the study conducted in Spain by Collado et al. (2010) in which *A. butzleri* was found more prevalent in sewage during spring (91.7 %) and summer (83.3 %) in Spain. Ghane (2014) also reported that *A. butzleri* was predominant in the spring and summer in the Caspian Sea with an occurrence rate of 3.04 and 2.28 %, respectively.

A. butzleri was the only species identified in all sample types. In a recent study (Šilha et al. 2015), the most frequent isolated species in wastewater was *A. cryaerophilus* (38.1 %). Collado et al. (2008) isolated species of *A. butzleri* (94 %), followed by *A. cryaerophilus* (30 %) and *A. skirrowii* (1.8 %) in environmental water samples. In another study, Morita et al. (2004) found that *A. butzleri* was the most frequent species isolated from river water samples in Japan and canal water samples in Thailand. Çelik and Ünver (2015) suggested that *A. cryaerophilus* and *A. skirrowii* may not be detected in environmental water or may be present under detection limit of methods due to the differences in ecological and biological characteristics of the organism. *A. butzleri* may indicate stronger viability than other species in water and may show competitive inhibitory effect on the other species present in population dynamic (Çelik and Ünver 2015).

As seen from Table 2, all *Arcobacter* species gave positive reaction for indoxyl-acetate hydrolysis and growth at 30 °C

Table 2 The biochemical characteristics of *Arcobacter* spp. isolated from water samples

	A. butzleri identified by m-PCR ($n = 21$)	*A. butzleri* identified by 16S rRNA sequencing ($n = 18$)	Unidentified ($n = 2$)
Indoxyl-acetate hydrolysis	21	18	2
Catalase activity			
Strong	1	4	–
Weak	20	14	2
H_2S production	–	–	–
Microaerophilic at			
30 °C	21	18	2
37 °C	19	13	2
42 °C	19	14	1
Aerobiosis at			
25 °C	21	18	2
30 °C	21	18	2
37 °C	19	13	2
Growth in/on			
2 % (*w*/*v*) NaCl	20	17	2
3.5 % (*w*/*v*) NaCl	14	16	2
0.04 % TTC	19	18	2
MacConkey agar	18	18	2

TTC 2,3,5-triphenyltetrazolium chloride

under microaerobic and aerobic conditions and at 25 °C during aerobic incubation and also failed in the production of H_2S. Most *Arcobacter* species (87.8 %) indicated weak catalase activity. On the other hand, they gave variable results during growth at 37 °C under both conditions. Also, there were differences in phenotypic patterns among species during growth in the presence of 2 and 3.5 % (*w*/*v*) NaCl and 0.04 % TTC and on MacConkey agar (Table 2). In total, 39, 32, and 39 of the 41 isolates grew in the presence of 2 and 3.5 % NaCl and 0.04 % TTC. And 38 strains were able to grow on MacConkey agar.

Conclusions

On the basis of the results of this study, it can be concluded that *A. butzleri* is a potential waterborne pathogen present in sewage, river, and spring water in Izmir. The presence of *A. butzleri* in the environmental water is of great importance for water quality and human health. Therefore, it is necessary to develop appropriate prevention and control strategies against *A. butzleri* in water sources to minimize public health risks.

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