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Isolation and determination of antimicrobial resistance of *Arcobacter* species isolated from animal faeces in the Diyarbakir region of Turkey using the 16S rDNA-RFLP method

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ABSTRACT: In this study, the presence of *Arcobacter* spp. was investigated in the faeces of cattle, sheep, goats, dogs and cloacal swab samples of chickens using the 16S rDNA-RFLP method. The prevalence of *Arcobacter* in these species was found to be 13%, 12%, 16%, 4% and 33%, respectively. On the other hand, *Arcobacter* spp. could not be isolated from rabbit faeces. A total of 78 (13%) *Arcobacter* spp. isolates were obtained from the 500 faecal samples and 100 cloacal swab samples examined in this study. From these 78 *Arcobacter* isolates, 24 (30.8%), 20 (25.6%), 11 (14.1%), 8 (10.7%), 4 (5.1%), 3 (3.9%) and 2 (2.6%) were identified by 16S rDNA-RLFP as *A. cryaerophilus*, *A. butzleri*, *A. skirrowii*, *A. cloacae*, *A. cibarius*, *A. halophilus*, and *A. nitrofigilis*, respectively. All *A. cryaerophilus* (n = 24) isolates were found to be resistant to cloxacillin; all *A. butzleri* (n = 20) and *A. skirrowii* isolates were found to be resistant to penicillin/novobiocin, cefoperazone, tetracycline and cloxacillin. It was determined in this study that clinically healthy cattle, sheep, goats, dogs and chickens are reservoirs of *Arcobacter* spp.

Keywords: cattle; sheep; goats; dogs; chickens; rabbits; antibiogram

Arcobacter spp. are S-shaped, gram-negative, spiral, motile, non-spore forming and fastidious microorganisms that belong to the *Campylobacteraceae* family. Their germination ability at 15 °C is the most significant distinctive feature of *Arcobacter* spp. that distinguishes them from the *Campylobacter* spp. (Kabeya et al. 2003).

Recently, the *Arcobacter* genus has been gaining increasing importance in community health due to its zoonotic potential, the appearance of new species and the fact that several species are emerging enteropathogens (Collado and Figueras 2011). Further, the genus has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications. The presence of *Arcobacter* spp. has been demonstrated in foods of animal origin, various animal faeces (such as cattle, sheep, dog, rabbit, chicken), the environment and water in many studies (Kabeya et al. 2003; Van Driessche et al. 2003; Vandenberg et al. 2006; Houf et al. 2008; De Smet et al. 2011; Shah et al. 2011; Noh et al. 2012; Suelam 2012). In their study, Figueras et al. (2012) increased the number of currently characterised *Arcobacter* spp. to 17. *Arcobacter* spp. lead to mastitis, abortus and gastrointestinal diseases in animals, and have been isolated from asymptomatic animals as well (Ho et al. 2006; Collado and Figueras 2011). Some *Arcobacter* spp. (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) have been isolated from the faeces of humans with diarrhoea or without diarrhoea, and sometimes from people with bacteraemia, endocarditis, and peritonitis (Shah et al. 2012).

To our knowledge, very little research has been performed on the genus *Arcobacter* in the study area, and since the public lacks information on the risks associated with this disease, the significance of *Arcobacter* spp. remains unclear. Therefore, in this

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study the prevalence and antimicrobial susceptibility of *Arcobacter* spp. was investigated in the faeces collected from healthy cattle, sheep, goats, dogs and rabbits, and in the cloacal swab samples of chickens using the 16S rDNA-RFLP method. All of the above represent potential sources of contamination for humans, the human food chain and other animals.

MATERIAL AND METHODS

Cloacal swabs and faecal samples. In this study, a total of 600 samples were collected from free-range farm animals in Diyarbakir province. The Diyarbakır province is located at 37°54'39''N 40°14'12''E and 660 m altitude. Average annual precipitation is 496 mm and its location on a map is shown in Figure 1. Chicken swab samples (n = 100) and 1 g samples of cattle (n = 100), sheep (n = 100), goat (n = 100), dog (n = 100) and rabbit faeces (n = 100) were collected at the same time from each animal; samples were immediately transferred at 5 ± 3 °C to the Veterinary Faculty at Dicle University in Diyarbakir and processed within one hour of collection.

Isolation media. *Arcobacter* enrichment broth was prepared using *Arcobacter* enrichment basal medium (Oxoid, CM965; Hampshire, UK) with cefoperazone-amphotericin-teicoplanin selective supplement (Oxoid, SR174E) as described by (Atabay and Corry 1998). Blood agar was prepared by adding 5% (v/v) defibrinated sheep blood in blood agar base No. 2 (Oxoid, CM271). Gas-generating kits (Anaerocult C, Merck, Germany) were used to provide a microaerobic atmosphere.

Method of isolation. Chicken swab samples and 1 g of cattle, sheep, goat, dog and rabbit faeces were homogenized in 20 ml of sterile distilled water and each suspension was then inoculated into 20 ml of double-strength *Arcobacter* enrichment broth. All enrichment media inoculated with the respective samples were incubated microaerobically at 30 °C for two days, as described by (Atabay and Corry 1998). After incubation, the enriched samples were plated onto non-selective blood agar using the membrane filtration method (Atabay and Corry 1998). A 47-mm diameter, 0.45 µ pore-size cellulose acetate membrane filter (Sartorius AG, Goettingen, Germany) was employed for the membrane filtration method. All the plates were incubated aerobically at 30 °C until visible growth was observed; plates were examined daily for up to 5–7 days. Suspected *Arcobacter* colonies with clear, small, gray-white, round colonies were tested for typical characteristic features by gram staining, growth under aerobic and microaerobic conditions at 30 °C, cellular morphology, catalase test, oxidase test, hippurate hydrolysis and for motility using the wet mount method. All suspected isolates were preserved in 20% glycerol at –80 °C for later identification using 16S rRNA PCR-RLFP.

Figure 1. Location of Diyarbakir province in Turkey

DNA extraction. DNA extraction was performed using the EZ-10 Spin Column Bacterial DNA Mini-Preps Kit (Bio Basic Inc., BS624, Canada) according to the manufacturer's instructions and extracted DNAs were stored at –20 °C until used. Primer sequences for 16S rRNA gene were taken from a previous study (Figueras et al. 2008). Primer sequences used are as follows: forward 5'-AACACATGCAAGTCGAACGA-3' and reverse 5'-GTCGTGAGATGTTGGGTTAA-3' (Figueras et al. 2012).

Identification of strains. For identification of *Arcobacter* spp., data from the study carried out by Figueras et al. (2012) were used. In this study, rRNAs of each known *Arcobacter* spp. were amplified by PCR and the amplicons were treated with several restriction enzymes. DNA band profiles of each strain were determined and used for classification. In our study, we used these DNA band profiles for distinguishing the strains from each other. Also, two reference strains, *A. butzleri* LMG 10828 and *A. cryaerophilus* LMG 10829, were included as positive controls.

PCR. PCR mixtures were prepared in 20 µl reaction volumes containing 1X Taq Buffer, 1.5 mM MgCl, 200 µM of each deoxynucleotide (dNTPs), 200 nM of each primer and 1.5 U Taq DNA polymerase (Thermo Scientific). Amplification was per-

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formed in an Arktik™ Thermal Cycler (Thermo Fisher Scientific Oy, Finland). PCR conditions were as follows: 5 min at 94 °C, followed by 30 amplification cycles, each consisting of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 90 s. Final extension were performed at 72 °C for 10 min. PCR products were separated on 1.5% agarose gels in 0.5 X TBE with the 100 bp GeneRuler DNA ladder (Thermo Scientific) and photographed. DNA fragments of 1026 bp were considered as positive for *Arcobacter* spp. Amplified PCR products were digested with FastDigest MseI restriction endonuclease at 65 °C for 10 min. Restricted fragments were separated using 15% polyacrylamide gel electrophoresis in 1 X TBE buffer at constant 20 mA with a 50 bp GeneRuler. Gels were stained with ethidium bromide and photographed (Figueras et al. 2012).

Determination of antimicrobial susceptibility. Antimicrobial susceptibility testing of the *Arcobacter* isolates was carried out using the Kirby-Bauer disc diffusion method. All isolates were cultured on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, followed by microaerobic incubation at 30 °C for 48 h. Bacterial colonies from fresh pure cultures were mixed with Mueller-Hinton broth; the turbidity of each inoculum was adjusted to McFarland 0.5 standards. Bacteria from each suspension were inoculated onto Mueller-Hinton agar using a sterile cotton-tipped swab. The plates were kept at 37 °C for 1–2 min to dry before antibiotic discs were dispensed. Incubation of the plates took place in a microaerobic atmosphere at 30 °C for 48 h and the diameter of the inhibition zones was measured with callipers. The susceptibility patterns (resistance/sensitivity) of the strains were determined according to the National Committee for Clinical Laboratory Standards (Shah et al. 2012).

RESULTS

Arcobacter spp.-positive isolates and their rates of prevalence in the faeces and cloacal swab samples (percentage) are presented in Table 1. A total of 600 samples were examined in this study and 78 (13%) *Arcobacter* spp. isolates were detected. These isolates were identified down to the species level using PCR followed by 16S rDNA-RFLP analysis. The highest number of *Arcobacter* spp. (six species) was observed in the goat faeces, and the lowest number was observed in the dog faeces (two species). The *Arcobacter* spp. isolated from the goat faeces were *A. cryaerophilus* (31.3%), *A. butzleri* (18.8%), *A. skirrowii* (12.5%), *A. cloacae* (12.5%), *A. nitrofigilis* (6.3%) and *A. halophilus* (6.3%); the *Arcobacter* spp. isolated from sheep faeces were *A. cryaerophilus* (33.3%), *A. skirrowii* (25%), *A. halophilus* (16.7%), *A. cibarius* (16.7%) and *A. butzleri* (8.3%); the *Arcobacter* spp. isolated from the cattle species were *A. butzleri* (38.5%), *A. cryaerophilus* (23.1%), *A. cloacae* (15.4%), *A. skirrowii* (7.7%) and *A. nitrofigilis* (7.7%); the *Arcobacter* spp. isolated from the cloacal swabs of the chickens were *A. butzleri* (30.3%), *A. cryaerophilus* (27.3%), *A. skirrowii* (15.2%), *A. cloacae* (12.1%) and *A. cibarius* (6.1%). Only *A. cryaerophilus* (75%) and *A. butzleri* (25%) spp. were identified in the dog faeces.

Table 1. Prevalence and distribution of *Arcobacter* spp. by PCR in various samples

Samples	Total sample number	Total positive samples	A.b.	A.c.	A.s.	A.n.	A.h.	A.cl.	A.cb.	A.b. + A.s.	A.b. + A.c.	A.c. + A.s.	A.b. + A.s. + A.c.
Cattle faeces	100	13	5	3	1	1	–	2	–	1	–	–	–
Sheep faeces	100	12	1	4	3	–	2	–	2	–	–	–	–
Goat faeces	100	16	3	5	2	1	1	2	–	–	1	–	1
Chicken cloacal swab	100	33	10	9	5	–	–	4	2	1	–	1	1
Dog faeces	100	4	1	3	–	–	–	–	–	–	–	–	–
Rabbit faeces	100	–	–	–	–	–	–	–	–	–	–	–	–
Total	600	78	20 (26%)	24 (31%)	11 (14%)	2 (3%)	3 (4%)	8 (10%)	4 (5%)	2 (2%)	1 (1%)	1 (1%)	2 (3%)

A.b. = *A. butzleri*, A.c. = *A. cryaerophilus*, A.s. = *A. skirrowii*, A.n. = *A. nitrofigilis*, A.h. = *A. halophilus*, A.cl. = *A. cloacae*, A.cb. = *A. cibarius*

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The resistance of the isolated *Arcobacter* spp. to various antimicrobial agents was investigated and the results are shown in Table 2. All of the *A. cryaerophilus* (*n* = 24) isolates were determined to be resistant to cloxacillin; all of the *A. butzleri* (*n* = 20) isolates were determined to be resistant to penicillin/novobiocin, cefoperazone, tetracycline and cloxacillin; all of the *A. skirrowii* (*n* = 11) isolates were determined to be resistant to penicillin/novobiocin, cefoperazone, tetracycline, ampicillin, cloxacillin and penicillin G.

DISCUSSION

Many recent studies have demonstrated the presence of *Arcobacter* spp. in animals or foods of animal origin (Van Driessche et al. 2003; De Smet et al. 2011; Shah et al. 2011; Ahmed and Balamurugan 2013). The prevalence of *Arcobacter* spp. in animals differs from country to country (Ho et al. 2006). The highest prevalence of *Arcobacter* spp. was detected in the faeces and rectal swab samples of clinically healthy cattle (Van Driessche et al. 2005). Kabeya et al. (2003) reported the *Arcobacter* spp. prevalence to be 3.6% in Japan, while Aydin et al. (2007) reported it to be 6.9% in Turkey, Van Driessche et al. (2003) found a prevalence of 39% in Belgium and Vilar et al. (2010) found one of 41.7% in Galicia. Kabeya et al. (2003) isolated *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* spp. Van Driessche et al. (2005) found that more than one *Arcobacter* spp. was present in 26% of the animals testing positive (11%). In our study, *Arcobacter* spp. were present in 13% of the samples of cattle faeces. In one of the positive samples (7.7%), *A. butzleri* and *A. skirrowii* were present together. It has been reported that more than one genotype may be found together due to the heterogeneity of the tested animals (Atabay et al. 2002; Van Driessche et al. 2005; Aydin et al. 2007; Shah et al. 2011). The most frequently isolated species in healthy livestock was *A. butzleri* (Kabeya et al. 2003; Van Driessche et al. 2003; Ongor et al. 2004). In our study, the dominant species was *A. butzleri* (38.5%) as well. This bacterium was followed by *A. cryaerophilus* (23.1%) and *A. cloacae* (15.4%). *A. nitrofigilis*

Table 2. Antibiotic susceptibility of *Arcobacter* strains isolated from cattle, sheep, goat, and dog faeces and from cloacal swabs of chicken according to CLSI standards

Antimicrobial agent, concentration (μg/disk)	*A. cryaerophilus* (*n* = 24)			*A. butzleri* (*n* = 20)			*A. skirrowii* (*n* = 11)		
	res	int	sus	res	int	sus	res	int	sus
Penicillin/Novobiocin (10 + 30 μg)	22	–	2	20	–	–	11	–	–
Ceftiofur (30 μg)	3	2	19	4	1	15	1	2	8
Cefoperazone (30 μg)	23	1	–	20	–	–	11	–	–
Cefoperazone (75 μg)	22	–	2	19	–	1	10	1	
Tetracycline (30 μg)	22	2	–	20	–	–	11	–	–
Gentamicin + Amoxicillin (7.4 + 27.6 μg)	4	2	18	4	2	14	2	–	9
Ampicillin (10 μg)	22	1	1	18	–	2	11	–	–
Amoxycillin (25 μg)	16	2	8	14	2	4	8	1	2
Erythromycin (15 μg)	15	1	8	12	2	6	7	–	4
Amoxicillin/clavulanic acid (20 + 10 μg)	8	4	12	6	3	11	2	2	7
Cloxacillin (5 μg)	24	–	–	20	–	–	11	–	–
Enrofloxacin (5 μg)	2	–	22	1	–	19	1	–	10
Penicillin G (10 μg)	22	2	–	19	1	–	11	–	–
Spiramycin (100 μg)	5	1	18	4	2	14	2	–	9
Doxycycline(30 μg)	5	2	17	2	–	18	2	–	9
Nalidixic acid (30 μg)	18	–	6	14	–	6	7	–	4
Streptomycin (25 μg)	6	–	18	6	–	14	3	1	7
Vancomycin (30 μg)	9	–	15	5	2	13	2	1	8

int = intermediate, res = resistant, sus = susceptible

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and *A. cloacae* were identified in cattle faeces for the first time in this study.

Aydin et al. (2007) failed to isolate *Arcobacter* spp. from the rectal swab samples obtained from 68 sheep in Turkey. In the study of Van Driessche et al. (2003) on 62 samples of sheep faeces, the presence of *Arcobacter* spp. was reported in 5% of samples by direct isolation and in 16% by enrichment. In the same study, only *A. cryaerophilus* was isolated using the direct isolation method, whereas *A. cryaerophilus* was isolated from eight samples and *A. butzleri* was isolated from two samples after the enrichment procedure. De Smet et al. (2011) isolated *Arcobacter* spp. from 66 of 153 sheep faecal samples (43.1%), and from 19 of 177 goat faecal samples (10.7%). Noh et al. (2012) reported the prevalence of *Arcobacter* spp. to be 15% in goat rectal swab samples in Malaysia. In our study, *Arcobacter* spp. were present in 12% of the sheep and 16% of the goat samples. *A. cryaerophilus* was the most frequently detected species among both the sheep and the goats. *A. halophilus* spp. in sheep and *A. halophilus*, *A. nitrofigilis* and *A. cloacae* spp. in goats were detected in this study for the first time. These species may originate from animal food, rangelands and water sources.

Different results were reported in studies on the presence of *Arcobacter* spp. in fowl. Gude et al. (2005) failed to isolate *Arcobacter* spp. from chicken faeces, while Aydin et al. (2007) could not isolate *Arcobacter* spp. from the cloacal swabs of broilers (n = 100) and layers (n = 100). Ho et al. (2008) reported isolation rates of *Arcobacter* spp. of between 3–85% from chicken intestines in five different flocks, but did not isolate any from cloacal swab samples. *Arcobacter* spp. were found at a rate of 14.5% in chicken cloacal swab samples in the study of Kabeya et al. (2003) in Japan. Patyal et al. (2011) reported the presence of *Arcobacter* spp. in rectal swab samples to be 14.7% by the classical method, and 17.3% by the PCR method, in a fowl farm in India. In the study of Atabay et al. (2006) in Denmark, the *Arcobacter* spp. positivity rate was found to be 72% in 29 chicken cloacal swab samples. In the same study, *A. butzleri* and *A. cryaerophilus* were isolated at rates of 45% and 31%, respectively. In this study, *Arcobacter* spp. were isolated from 33% of the chicken cloacal swab samples. The most frequently isolated species were *A. butzleri* (30.3%), *A. cryaerophilus* (27.3%) and *A. skirrowii* (15.2%). These high isolation rates support the finding that fowl are an important reservoir of *Arcobacter* spp. (Atabay et al. 2006). Houf et al. (2005) isolated *A. cibarius* from broiler carcasses for the first time. *A. cloacae* and *A. cibarius* spp. were first detected in chicken cloacal swab samples in this study. We suggest that animal food and environmental contamination may be the sources of these species.

Aydin et al. (2007) failed to isolate *Arcobacter* spp. from rectal swab samples obtained from 62 dogs in Turkey. Houf et al. (2008) reported the presence of *Arcobacter* spp. in only five out of 267 samples of dog faeces (1.9%). In this study, *Arcobacter* spp. were detected in 4% (4/100) of the dog faeces. In this study, 75% of the isolated *Arcobacter* spp. were *A. cryaerophilus* and 25% were *A. butzleri*, whereas these rates were 80% and 20%, respectively, in the study of Houf et al. (2008).

Suelam (2012) reported the presence of *Arcobacter* spp. in all 20 (100%) samples of rabbit faeces, and the identified species were *A. cryaerophilus* (45%), *A. skirrowii* (40%) and *A. butzleri* (15%). In this study, no *Arcobacter* spp. were isolated from any of the 100 samples of rabbit faeces analysed. This difference may be explained by the fact that both studies were limited to certain farms only. Studies conducted with larger sample sizes and covering wider areas are needed.

Different studies report different rates of detection of *Arcobacter* spp. in animal faeces. It is believed that these differences are derived from farming practices (hygiene, feeding), age, geographical location (Golla et al. 2002), study design, sample size, sampling method, isolation methods (Van Driessche et al. 2005), environment and water sources (Ho et al. 2006).

Except for *Arcobacter butzleri*, *A. cryaerophilus* and *A. skirrowii* (Shah et al. 2012), that are reported to be pathogenic for both animals and humans, food and environment-related species have been reported as well. Isolation and identification of *A. cibarius* from chicken meat (Houf et al. 2005), *A. cloacae* from mussels and sewage (Levican et al. 2013), *A. halophilus* from a hypersaline lagoon (Donachie et al. 2005) and *A. nitrofigilis* from the roots of *Spartina alterniflora* (McClung et al. 1983) have all been reported.

We believe that the isolation of new *Arcobacter* spp. in our study was the result of employing the MseI enzyme before the application of TaqI and DdeI restriction enzymes after the PCR stage.

The susceptibility of *Arcobacter* spp. to antibiotics is important with regard to the development of

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nutritional media used for isolation, and with regard to the treatment of diseases observed in animals and humans (Unver et al. 2013). *Arcobacter* spp. isolated from different sources such as humans, winged carcasses, meat and the environment have been shown to have multi-drug resistance. The results of our study support this finding. In the study of Unver et al. (2013) on domestic geese in Kars, Turkey, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* spp. were reported to be resistant to cloxacillin and ampicillin, and susceptible to enrofloxacin. These findings are in agreement with the results of our study. However, susceptibility to oxytetracycline and erythromycin was detected in the same investigation in contrast to our study. In the study of Vandenberg et al. (2006), 78.7% of the *A. butzleri* isolates were found to be susceptible to ampicillin and erythromycin, and all (100%) were susceptible to tetracycline. In the studies of Atabay and Aydin (2001) on samples from broiler chickens, and of Kabeya et al. (2004) on beef, pork and chicken meat in Japan, the *Arcobacter* spp. isolates were found to be susceptible to tetracycline. In contrast to the results of other studies, the isolates obtained in this study were found to be susceptible to important antibiotics used in the treatment of *Arcobacter* spp. infections both in humans and animals. The tetracycline, ampicillin and erythromycin resistances of *A. butzleri* were found to be 100%, 90% and 60%, respectively; in *A. cryaerophilus* these values were found to be 91.7%, 91.7% and 62.5%, respectively; and in *A. skirrowii* the rates of resistance were found to be 100%, 100% and 63.6%, respectively. In the study of Shah et al. (2012), the rates of tetracycline resistance of *A. butzleri* were found to be only 6.5% (M.I.C.E.) and 8.7% (disc diffusion). Houf et al. (2004) reported that resistance to erythromycin and ciprofloxacin, used in the treatment of infections caused by *Campylobacter* spp. and related bacteria, has been increasing. These findings support our results. Rates of resistance and of susceptibility have been linked to differences between antimicrobial agents, the antibiotics used, *Arcobacter* spp. isolates and the sources of these isolates.

The highest number of *Arcobacter* spp. isolates was found in chicken swab samples (33/100), followed by samples of goat (16/100), cattle (13/100), sheep (12/100) and dog faeces (4/100). These animal species are regarded as reservoirs for *Arcobacter* spp. that cause animal health and community health risks by contaminating animal food. *Arcobacter* spp. was not detected in samples of rabbit faeces. The *A. butzleri, A. cryaerophilus* and *A. skirrowii* spp. – the most frequently isolated species in this study – are proven to cause various diseases in both humans and animals. Further studies are needed to evaluate the pathogenicity of other species (*A. nitrofigilis*, *A. halophilus*, *A. cloacae*, *A. cibarius*) for animals and humans.

In the present study, the prevalence rate of *Arcobacter* spp. was found to be 13%. The dominant species was *A. butzleri* (38.5%). This species was followed by *A. cryaerophilus* (23.1%) and *A. cloacae* (15.4%). Our positive rates are lower than those reported previously. The *Arcobacter* species isolated in this study that are known to be pathogenic in humans were resistant to cloxacillin, tetracycline, ampicillin and erythromycin. This should be considered when treatment options are evaluated.

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