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ELMALI, Mehmet; Yeim CAN, Hayriye
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Occurence and antimicrobial resistance of *Arcobacter* species in food and slaughterhouse samples

Mehmet ELMALI[1], Hayriye Yeşim CAN[1]*

Abstract

The objectives of this study were: i) to isolate *Arcobacter* species (*Arcobacter butzleri*, *Arcobacter skirrowii, Arcobacter cryaerophilus*) from different foods and sources, ii) to verify the isolates by multiplex PCR assay, iii) to detect the antibiotic resistance profiles of the isolates. In this study a total of 60 *Arcobacter* isolates were obtained. *Arcobacter* species were mostly isolated from swab samples (40%), followed by wastewater (29.1%), broiler wing meat (30%), raw milk (23.9%) and minced meat (6.6%). Regarding the seasonal distribution of *Arcobacter* from swab and wastewater samples, the bacterium was commonly isolated from wastewater in winter and spring, while it was frequently detected in swab samples during autumn and spring. All of the isolates were found to be resistant to nalidixic acid, ampicillin, rifampin, and erythromycin. The most effective antibiotic was tetracycline, because 96.66% of the isolates were susceptible against it. This is the first report of the isolation, seasonal distribution and antimicrobial susceptibility of *Arcobacter* species in cattle slaughterhouse samples in Turkey. These results indicate that foods of animal origin and cattle slaughterhouses are significant source of the antimicrobial resistant arcobacters.

Keywords: *Arcobacter*; antimicrobial resistance; raw milk; minced meat; wastewater.

Practical Application: To reveal the presence of *Arcobacter* species in foods and cattle slaughterhouse samples.

1 Introduction

Arcobacter are an important pathogenic bacteria known as zoonosis. They are aerotolerant, Gram-negative bacteria that can grow under both aerobic and microaerophilic conditions, and optimally grow at 30 °C. They are motile and most strains are non-hemolytic. *Arcobacter* is one of the genera within the family *Campylobacteraceae*. *Arcobacter butzleri, Arcobacter cryaerophilus, Arcobacter skirrowii, Arcobacter nitrofigilis, Arcobacter cibarius* and *Arcobacter halophilus* are the most well known species in the genera (Fernandez et al., 2015; Giacometti et al., 2015a; Harmon & Wesley, 1996; Van den Abeele et al., 2014).

Some researches have reported that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been associated with human and animal infections. These species are considered as emerging pathogens and have been isolated from patients with bacteraemia, endocarditis, peritonitis and diarrhea. They cause abortion, mastitis and diarrhea in farm animals. Colonised healthy animals are also an important source of *Arcobacter* species (Alonso et al., 2014; Collado et al., 2014; Corry & Atabay, 2001; Girbau et al., 2015; Houf et al., 2000).

Arcobacters are transmitted by food and water. Especially since the 2000s studies conducted on this subject came into prominence in the world. After this term, *Arcobacter* species have been isolated from different foods and sources such as raw milk, cheese, chicken, pork and beef meats, water, sewage water, seafood and fecal samples in many countries including Turkey (Akıncıoglu, 2011; Atabay et al., 2003; Aydin et al., 2007; Çelik & Ünver, 2015; Ertas et al., 2010; Yesilmen et al., 2014), Iran (Khoshbakht et al., 2014; Rahimi et al., 2012), Italy (Giacometti et al., 2015a, b), Costa Rica (Bogantes et al., 2015), Germany (Lehmann et al., 2015), India (Ramees et al., 2014; Verma et al., 2015), Czech Republic (Šilha et al., 2015), Poland (Zacharow et al., 2015), Spain (Alonso et al., 2014), Belgium (Van den Abeele et al., 2014).

Limited information about the presence and antimicrobial susceptibility profiles of *Arcobacters* in foods and cattle slaughterhouse sources is available in Turkey. Therefore, in this study, we aimed to determine the prevalence of *Arcobacter* species in some foods (raw milk, broiler meat, minced meat) and cattle slaughterhouse samples (wastewater, swab), and to investigate the antimicrobial resistance profiles of the isolates.

2 Materials and methods

2.1 Sample collection

In this study, a total of 229 various samples were analysed. Samples including 40 frozen broiler wing meat, 45 minced beef meat samples, and 46 raw cow milk were collected in Hatay Region, Turkey. Between June 2014-May 2015, 50 swabs taken from the tools and equipment (e.g. knives, meat chopping boards) in cattle slaughterhouses, and 48 slaughterhouse wastewater discharged after cutting process were also used as materials in the study. The slaughterhouse samples were collected during

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[1] *Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Mustafa Kemal University, Antakya, Hatay, Turkey*
*Corresponding author: yesimcan@mku.edu.tr

every month. The samples were immediately transported to the laboratory in a cool box for analysis.

2.2 Isolation media

For isolation *Arcobacter* spp., Arcobacter Enrichment Broth (AEB; Oxoid, Basingstoke, Hampshire, England), BBL™ Blood Agar Base (BA; Le Pont de Claix, France), and Campylobacter Blood-Free Base Bolton (mCCDA; Biolife, Milano, Italia) were used. AEB was used with adding cefoperazon-amphotericin B-teicoplanin (CAT) selective supplement (Oxoid). BA was used with adding 5% defibrinated sheep blood. mCCDA was prepared with using CCDA selective supplement (Oxoid). All of isolation mediums were used together with gas generating kits (Oxoid) for making a microaerophilic condition for *Arcobacter* isolation (Atabay & Corry, 1997).

2.3 Membrane filtration technique

For the isolation of *Arcobacter*, we used membrane filtration technique. After preenrichment, 300 µL of each enriched samples were plated onto BA using cellulose acetate membrane filters (Filterlab, Barcelona, Spain) with a pore size of 0.45 µM according to the Atabay et al. (2003). The filters were removed after 30 min, and the inoculum passing through the filter was streaked on the agar by using loop. Then, the plates were incubated microaerobically at 30 °C for 48-72 h. Suspect colonies were sub-cultured on mCCDA and incubated microaerobically at 30 °C for 48-72 h. Opaque and whitish-gray colonies were selected for DNA extraction.

Isolation of Arcobacter from minced beef meat

In this study, we modified isolation prodecure reported by Aydin et al. (2007) as below. Ten grams of minced beef meat samples were added to 90 mL of AEB, and homogenised with stomacher (Bagmixer400, Interscience, France) for 2 min. Then, the homogenates were incubated in microaerophilic conditions at 30 °C for 48 h for preenrichment.

Isolation of Arcobacter from broiler wing meat

In the present study, we made some changes to isolation method for broiler meat reported by Aydin et al. (2007). Twenty five grams of broiler wing meat samples were suspended with 225 mL AEB, and homogenised for 2 min. Then, they were incubated microaerobically at 30 °C for 48 h for preenrichment.

Isolation of Arcobacter from raw milk

Preenrichment procedure of Atabay et al. (2003) for milk samples was performed with some modifications. AEB was added onto 20 mL of each raw milk sample, mixed throughly, and incubated microaerobically at 30 °C for 48 h for preenrichment.

Isolation of Arcobacter from swabs

We modified preenrichment procedure for swab samples described by Aydin et al. (2007). Twenty mililiters of AEB was added to swab samples, mixed throughly, and incubated microaerobically at 30 °C for 48 h for preenrichment.

Isolation of Arcobacter from wastewater samples

Slaughterhouse wastewater samples (20 mL) were added to 20 mL of AEB, mixed throughly, and incubated microaerobically at 30 °C for 48 h for preenrichment. The isolation procedure was carried out according to the Aydin et al. (2007).

2.4 Verification of Arcobacter spp. by PCR analysis

DNA extraction was performed using a Bacterial DNA Extraction kit (Nucleic Acid Extraction Kit, GF-1, Vivantis, Malaysia), following the kit manufacturer's instructions and extracted DNA samples were stored at –20 °C until the PCR analysis.

For *Arcobacter* spp., primers described by Harmon & Wesley (1996) were used. PCR mixture of Akıncıoğlu (2011) was performed in a total volume of 30 µL containing 2 µL of template DNA, 2.5 µL of *Taq* 10xbuffer (Thermoscientific, Lithuania), 1.5 mM $MgCI_2$ (Thermoscientific), 0.2 mM deoxynucleoside triphosphate mixture (EURx, Poland), 0.16 µM of primers (Ella Biotech, Germany), and 1.5 U *Taq* DNA polymerase (Thermoscientific). PCR amplification conditions were carried out as below: initial denaturation at 94 °C for 4 min, followed by 29 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min (Akıncıoğlu, 2011; Harmon & Wesley, 1996).

2.5 Identification of Arcobacter spp. by multiplex PCR analysis

To detect *A. butzleri*, *A. skirrowii,* and *A. cryaerophilus*, specific primer pairs described by Houf et al. (2000) were used. For the verification of *A. butzleri*, *A. skirowii*, and *A. cryaerophilus*, PCR master mix was prepared with the same concentrations as indicated above. Akıncıoğlu (2011)'s DNA amplification protocol was carried out with an initial denaturation of 94 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. Final extension cycle was performed at 72 °C for 7 min. Amplified PCR products were detected by electrophoresis in %1.5 agarose (Sigma-Aldrich, St Louis, USA) at 100 V for 1 h (CS-300V, Cleaver Scientific, England). The bands were then visualised under a UV transilluminator (UVP, Upland, USA).

2.6 Antimicrobial susceptibility

Antimicrobial susceptibility testing of the *Arcobacter* isolates against 9 antibiotics was performed on Mueller-Hinton agar (Oxoid) by the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015). Tested antibiotics were as follows: erythromycin (15 µg/disc), nalidixic acid (5 µg/disc), ampicillin (10 µg/disc), tetracycline (30 µg/disc), iminepem (10 µg/disc), gentamicin (10 µg/disc), ciprofloxacin (5 µg/disc), rifampin (5 µg/disc), azithromycin (15 µg/disc). The *Arcobacter* isolates were sub-cultured on BA and incubated at 30 °C for 48 h. Colonies were then suspended in saline solution (0.5%) for adjusting the turbidity of the suspension to MacFarland 0.5 standard. Each suspension was inoculated on Mueller-Hinton agar with a sterile cotton swab. The plates were

incubated aerobically at 30 °C for 48-72 h. Then, the diameters of inhibition zones were measured and the isolates were classified as susceptible, intermediate resistant, and resistant.

3 Results

3.1 Contamination with Arcobacter species

In this study, sixty (26.2%) of the samples were found to be contaminated with *Arcobacter*. *Arcobacter* spp. were isolated from 23.9%, 30%, 6.6%, 29.1% and 40% of raw milk, broiler wing meat, minced meat, wastewater, and swab samples, respectively.

A total of 60 *Arcobacter* isolates were obtained in this study, and isolates were identified at species level using multiplex PCR (Figure 1). Among these isolates, 23 were identified as *A. butzleri*, one was *A. skirrowii*, and six were *A. cryaerophilus*. Of these isolates, 11 were also detected as *A. butzleri* + *A. skirrowii* + *A. cryaerophilus*, 7 were *A. butzleri* + *A. cryaerophilus*, 3 were *A. cryoaerophilus* + *A. skirrowii* and 4 were *A. butzleri* + *A. skirrowii*. Five of these isolates were determined as other *Arcobacter* species (Table 1). Also, in this study, the most prevalent species was *A. butzleri* at a level of 38.3% (23/60), followed by 10% (6/60) *A. cryaerophilus* and 1.6% (1/60) *A. skirrowii*. *A. butzleri* was more frequently recovered from wastewater samples (78.5%), followed by broiler meat (41.6%), minced meat (33.3%), and swab samples (30%). *A. skirrowii* was only determined in minced meat. *A. cryaerophilus* was more commonly isolated from raw milk (36.3%) and broiler meat (16.6%) in the present study.

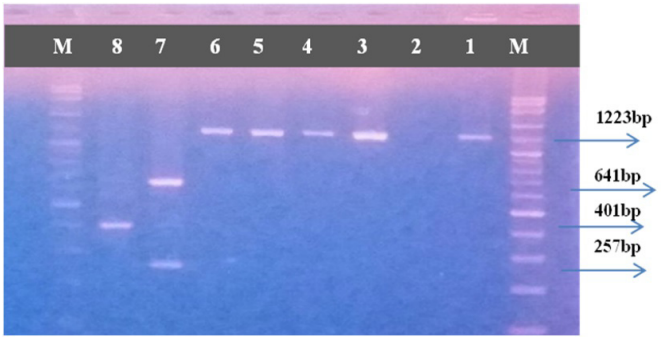

Figure 1. Agarose gel electrophoresis of *Arcobacter* spp.; *A. butzleri; A.skirrowii* and *A. cryaerophilus*. Lane M, 100 bp plus DNA ladder (Bioron, Germany); Lane 1, positive control; Lane 2, negative control; Lane 3-6, *Arcobacter* spp.; Lane 7: *A. skirrowii* + *A. cryaerophilus*; Lane 8: *A. butzleri*.

3.2 Seasonal and monthly distribution of the isolates from swab and wastewater samples

In the present study, a total of 98 cattle slaughterhouse samples including 50 swabs and 48 wastewater were collected during every month. Then, the samples were tested for the presence of *Arcobacter* species and the results were evaluated according to the months and seasons. Overall, according to the monthly and seasonal distributions of the isolates from wastewater samples, the contamination level was found to be 10.4% (5/48) in winter and spring, while it was 4.1% (2/48) in summer and autumn. In addition, all of the wastewater samples collected during December were found to be contaminated with *Arcobacter* spp (Table 2).

In the evaluation of the isolates recovered from swab samples according to the months and seasons, *Arcobacter* was not detected in any of the samples collected during winter. *Arcobacter* spp. was recovered from the swab samples with a rate of 8% (4/50), 14% (7/50), and 18% (9/50) in summer, spring, and autumn, respectively (Table 2). Also, a total of six swab samples were taken only in November and *Arcobacter* was detected in all of them.

3.3 Antimicrobial susceptibility

Now, there are no zone diameter interpretive criteria for comparison of the antibiotic resistance level of *Arcobacter* spp., so that the antibiotic susceptibility results of the isolates for nalidixic acid, ampicillin, tetracycline, imipenem, gentamicin, ciprofloxacin and azithromycin were compared with *Enterobactericeae* standards, while resistance to rifampin and erythromycin were evaluated according to *Staphylococcus* spp. standards of CLSI (2015). The results are shown in Table 3.

As a result of antibiotic susceptibility test, all of the *Arcobacter* isolates were found to be resistant to nalidixic acid, ampicillin, rifampin, and erythromycin. Resistance to ciprofloxacin gentamicin, and azithromycin was found to be 16.66%, 21.66%, and 23.33% among the isolates, respectively. The most effective antibiotic was tetracycline, because 96.66% of the isolates were susceptible against it. Resistance to imipenem was found very high (96.66%) among the isolates. In this study, all of the isolates were found to be resistant to one or more antibiotics tested.

Table 1. Distribution of *Arcobacter* species in analysed samples.

Samples	N of samples	*Arcobacter* spp.	*A.b*	*A.s*	*A. c*	*A. b* + *A. s* + *A. c*	*A. b*+ *A. c*	*A. c* + *A. s*	*A. b* + *A. s*	Other *Arcobacter* spp.
Raw milk	46	11 (23.9%)	0	0	4(36.3%)	5 (45.4%)	0	1 (9.0%)	0	1 (9.0%)
Broiler meat	40	12 (30%)	5 (41.6%)	0	2(16.6%)	0	3 (25%)	0	0	2 (16.6%)
Minced meat	45	3 (6.6%)	1 (33.3%)	1(33.3%)	0	0	0	0	1 (33.3%)	0
Waste water	48	14 (29.1%)	11 (78.5%)	0	0	2 (14.2%)	0	0	0	1 (7.1%)
Swab	50	20 (40%)	6 (30%)	0	0	4 (20%)	4 (20%)	2 (10%)	3 (15%)	1 (5%)
Total	229	60 (26.2%)	23 (38.3%)	1 (1.6%)	6 (10%)	11 (18.3%)	7(11.6%)	3 (5%)	4 (6.6%)	5 (8.3%)

A.b: *A. butzleri*; A.s: *A. skirrowii*; A.c: *A. cryaerophilus*.

Table 2. The monthly/seasonal distribution of *Arcobacter* isolated from swab and wastewater samples.

Samples	Sampling period											
	Summer (2014)			Autumn (2014)			Winter (2014-2015)			Spring (2015)		
	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May
Wastewater (n = 14)	2	0	0	0	2	0	4	0	1	0	3	2
Swab (n = 20)	3	0	1	1	2	6	0	0	0	2	2	3
Total (n =34)	5	0	1	1	4	6	4	0	1	2	5	5

n: number of isolates.

Table 3. Antimicrobial resistance profile of *Arcobacter* isolates (n = 60).

Antimicrobials	Resistant *n* (%)	Intermediate *n* (%)	Susceptible *n* (%)
Nalidixic acid	60 (100)	0	0
Ampicillin	60 (100)	0	0
Tetracycline	2 (3.33)	0	58 (96.66)
Imipenem	58 (96.66)	1 (1.66)	1 (1.66)
Gentamicin	5 (8.33)	8 (13.33)	47 (78.33)
Ciprofloxacin	4 (6.66)	6 (10)	50 (83.33)
Azithromycin	14 (23.33)	0	46 (76.66)
Rifampin	60 (100)	0	0
Erythromycin	60 (100)	0	0

4 Discussion

When we examine studies conducted in Turkey regarding the determination of contamination levels of animal food products with *Arcobacter*, we can see that Atabay et al. (2003) found 65.3% contamination on chicken carcasses, while Aydin et al. (2007) found 68%, 4% and 37% contamination in chicken meat, turkey meat, and minced meat, respectively. Consistent with these studies, *Arcobacter* was isolated most frequently from broiler meat among the food samples analysed in our study. Again supporting these findings, Corry & Atabay (2001) have reported very high levels of *Arcobacter* spp. contamination in poultry meat. Similar to studies carried out by Aydin et al. (2007), Ertas et al. (2010) and Yesilmen et al. (2014) most identified species in the samples was *A. butzleri*. In some samples, they also found *Arcobacter* species as parts of a mixed culture. In our study, the level of *Arcobacter* spp. contamination in raw milk was found to be 23.9%. Our finding was lower than the contamination level (36%) in the study of Yesilmen et al. (2014), but it was high as compared with the study (6%) of Ertas et al. (2010).

In other studies, Çelik & Ünver (2015), Akıncıoğlu (2011) detected *Arcobacter* spp. with a rate of 12.3% and 37%, respectively in various water sources. While Aydin et al. (2007), Çelik & Ünver (2015) found no *Arcobacter* in drinking water samples, Ertas et al. (2010) and Akıncıoğlu (2011) found the bacteria in drinking water. In this study, we found high levels of *Arcobacter* spp. contamination in slaughterhouse wastewater samples (29.1%) and in swab samples taken from meat cutting boards and knives (40%). *A. butzleri* was the predominant species isolated from the cattle slaughterhouse samples. In addition, we've observed that the slaughterhouse samples taken in spring and autumn have more contamination of the bacteria. To our knowledge, this study is the first report in this regard in Turkey. Considering these results, we think that meat cutting boards and knives may constitute a potential risk for the contamination of red meat with *Arcobacter*.

When we examine researches conducted in other countries in this regard, we can see that Zacharow et al. (2015) and Khoshbakht et al. (2014) found contamination of meat samples with *Arcobacter* to be much higher than our study (40.4% and 45% respectively, whereas our study had 17.6% contamination). While Bogantes et al. (2015) observed a low prevalence of *Arcobacter* in poultry (11%), Kabeya et al. (2004) and Rahimi et al. (2012) determined a high prevalence of *Arcobacter* in poultry, which is similar to our study. In accordance to above studies, the predominant species was *A. butzleri* in the meat samples. While Verma et al. (2015) found the level of *Arcobacter* contamination in various animal products and environmental samples as 11.3% lower than our study, Šilha et al. (2015) found a contamination level of 36.8%, higher than our findings. Similarly, Verma et al. (2015) detected *A. butzleri, A. skirrowii*, and *A. cryaerophilus* as parts of a mixed culture in some samples. Giacometti et al. (2015b) determined *Arcobacter* at a level of 22.6% in samples taken from a dairy farm and similarly found that *A. cryaerophilus* was the predominant species in the milk. Lehmann et al. (2015) found *Arcobacter c*ontamination of fish meat, poultry and minced meat as 34%, 26.8% and 2%, respectively. In Chile, studies of Collado et al. (2014) and Fernandez et al. (2015) have shown that *A. butzleri* was the most frequently isolated species also in sea products.

As a result, it can be seen that foods of animal origin, sea products and water sources are contaminated with *Arcobacter* and contamination levels are varied. This variation may be a result of the fact that studies were conducted in different regions, and that isolation methods used have different sensitivity levels.

There is limited information about the antimicrobial susceptibility of *Arcobacter*. In line with our findings, Collado et al. (2014), Akıncıoğlu (2011) found high resistance to nalidixic acid and ampicillin among the *Arcobacter* strains. Akıncıoğlu (2011), Yesilmen et al. (2014), Zacharow et al. (2015) and we found high resistance to erythromycin, different from Collado et al. (2014) and Kabeya et al. (2004). Only four isolates were resistant and six isolates were intermediately resistant to ciprofloxacin in the present study.

Yesilmen et al. (2014) reported that the acquired resistance to tetracycline and ampicillin among the foodborne isolates

may raise concern for the treatment of *Arcobacter* infections in humans and animals, since both of these antibiotics are generally prefered. In this study, susceptibility to tetracycline was very high among the isolates. Zacharow et al. (2015), Akıncıoğlu (2011), Kabeya et al. (2004), Atabay & Aydin (2001) found *Arcobacte*r strains susceptible to tetracycline in contrast to Yesilmen et al. (2014).

In conclusion, our results show that the presence of *Arcobacter* species in foods of animal origin and cattle slaughterhouse samples. Because of this, raw milk, minced meat, broiler meat and also knives, meat chopping boards and wastewater in cattle slughterhouse are considered to be an important source of *Arcobacter* species. At the same time, all of *Arcobacter* isolates recovered from these samples were found to be resistant to one or more antibiotics tested. Therefore, these results should be taken into account when controlling the contamination with *Arcobacter* and also treating the infections caused by *Arcobacter* species in animals and humans.

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