Comparative Immunology, Microbiology and Infectious Diseases 44 (2016) 37–40

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Occurrence and antimicrobial resistance of emergent *Arcobacter* spp. isolated from cattle and sheep in Iran

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ARTICLE INFO

Article history:
Received 8 February 2015
Received in revised form
28 November 2015
Accepted 2 December 2015

Keywords:
Arcobacter
Cattle
Sheep
Antimicrobial susceptibility

ABSTRACT

This study is conducted to determine the occurrence and antimicrobial resistance of *Arcobacter* spp. isolated from clinically healthy food animals. A total of 308 samples from cattle (200) and sheep (108) were collected from Shiraz slaughterhouse, southern Iran to investigate the presence of the important *Arcobacter* spp. using cultivation and Polymerase Chain Reaction (PCR) methods. Antimicrobial susceptibility of *Arcobacter* isolates was determined for 18 antibiotics using disk diffusion method. Among 308 samples, 27 (8.7%) and 44 (14.28%) were positive for the presence of *Arcobacter* species with cultivation and PCR procedures, respectively. The predominant species was *A. butzleri* in both cattle (58.33%) and sheep (55%). In addition, concurrent incidence of the species was observed in 25% of the positive samples. All *Arcobacter* isolates were resistant to rifampicin, vancomycin, ceftriaxone, trimethoprim and cephalothin. The isolates showed high susceptibility to tetracycline, oxytetracycline, erythromycin, ciprofloxacin, kanamycin, amikacin, gentamicin and enrofloxacin. No significant difference among cattle and sheep isolates in resistance pattern was observed. The results indicate that cattle and sheep are significant intestinal carriers for *Arcobacter* spp. Moreover, tetracycline and aminoglycosides showed great effects on *Arcobacter* species in antibiogram test and can be used for treatment of human *Arcobacter* infections.

1. Introduction

Arcobacter is a member of the Epsilobacteria group, which includes the genus *Campylobacter* and *Helicobacter* [1]. Until now, three species of *Arcobacter* spp., *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, have been associated with a variety of human and animal diseases [1–3]; however, they have been isolated from healthy livestock [4]. Regarding *Arcobacter* species detection in human and animal diseases and meat products, *A. butzleri* has been the most frequently detected species [5–7]. *Arcobacter* species have been frequently found in feces and rectal swabs of clinically healthy cattle [8] and sheep [5]. The abundant presence of *A. butzleri, A. cryaerophilus* and *A. skirrowii* in food animals and drinking water and their association with human and animal diseases lead to consideration of these bacteria as significant foodborne and waterborne agents [3,5,8]. Food contamination with antibiotic-resistant bacteria can be a major threat to public health; because the antibiotic resistance determinants can be transferred to other pathogenic bacteria, potentially compromising the treatment of severe bacterial infections [9]. Although *Arcobacter* infections are normally self-limited and do not require antimicrobial drugs, the most commonly prescribed drugs in cases with severe or prolonged symptoms are erythromycin or fluoroquinolones such as ciprofloxacin [10]. Tetracycline, doxycycline, and gentamicin are sometimes listed as alternative drugs for treatment [11]. Contamination of foods of animal origin is assumed to occur during slaughter process [4]. Due to these reasons, accurate information about the prevalence of these potentially pathogenic bacteria in food animals is essential to consumer's safety. Currently, the data about the prevalence of *Arcobacter* in food animals like sheep and cattle in developing countries, particularly Iran, is indistinct. In addition, reports on antimicrobial resistance patterns in *Arcobacter* spp. isolated from cattle and sheep are lacking. Therefore, the present study was

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http://dx.doi.org/10.1016/j.cimid.2015.12.002
0147-9571/

conducted to determine the occurrence and antimicrobial resistance of *Arcobacter* spp. isolated from sheep and cattle in southern Iran.

2. Materials and methods

2.1. Sampling

A total of 308 fecal samples were collected from apparently healthy sheep (108) and cattle (200) in an industrial abattoir in Fars province, southern Iran during the period of April to July 2012 over 10 visits. The abattoir received cattle and sheep from different herds within and outside the province. Fecal samples were collected randomly from the rectum of apparently healthy animals prior to slaughter using sterile gloves, placed into sterile whirl-pack bags, conveyed to the microbiology laboratory, Faculty of Veterinary Medicine, Shiraz University, (Shiraz, Iran), in special ice-filled containers within 4 h of sampling.

2.2. Isolation of Arcobacter spp.

The fecal samples were enriched and cultured by the procedure of Rahimi et al. [12] with slight modifications according to Ferreira et al. [13]. Briefly, the samples were inoculated into Arcobacter broth (Oxoid, Hampshire, England) with cefoperazone, amphotericin B, teicoplanin (CAT) selective supplement (Oxoid, Hampshire, England) added and incubated microaerobically for 72 h at 30 °C. From each enriched sample, 50 μL was dropped on a cellulose-nitrate membrane filter (0.65 μm) placed on the selective blood agar plates (Brain heart infusion agar supplemented with 5% (v/v) defibrinated sheep blood and CAT selective supplement). After 1 h incubation at 30 °C, in aerobic atmosphere, the membrane filters were removed and filtrates evenly spread over the agar surface. The plates were incubated for 48 h at 30 °C under microaerophilic conditions. Suspicious bacterial colonies (clear white and/or gray pinpoint colonies) were picked, purified by subculture and identified according to Atabay et al. [14]. The identified isolates were confirmed by 16SrRNA using specific primers described by González et al. [15].

2.3. DNA preparation and PCR assay

Bacterial DNA was extracted from both isolates and enriched samples by the procedure of Khoshbakht et al. [16] using phenol-chloroform technique. The purity and concentration of the DNA were estimated by spectrophotometry at 260 and 280 nm. Genus and species-specific PCR reactions were performed for identification of *Arcobacter* genus [15], *A. butzleri* [17], *A. cryaerophilus* and *A. skirrowii* [18] species, respectively by proper primers (Table 1). The PCR reaction mixtures consisted of 2 μL of the DNA template, 2.5 μL 10× PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $[NH_4]_2SO_4$), (CinnaGen, Iran), 1 μL dNTPs (50 mM), (CinnaGen, Iran), 1 μL (1 U Ampli *Taq* DNA polymerase), (CinnaGen, Iran), 1 μL (25 pmol) from the forward and reverse primers (CinnaGen, Iran), shown in Table 1. The volume of each reaction mixture was adjusted to 25 μL using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was adjusted under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, and annealing as shown in Table 1 for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 10 min and the PCR products remained in the thermal cycler at 4 °C until they were collected. Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The 100 bp DNA ladder (CinnaGen, Iran) was used as molecular size marker and visualization was undertaken using a UV transilluminator.

2.4. Antimicrobial susceptibility testing

The *Arcobacter* isolates were examined for susceptibility to 18 commercially available antibiotic disks using the disk diffusion technique. Each fresh culture of *Arcobacter* isolate was suspended in 0.85% (w/V) sterile NaCl and adjusted to a turbidity of 0.5 McFarland. Each suspension was inoculated with a sterile swab onto 150 mm diameter Mueller-Hinton agar plate (Oxoid, Hampshire, England) supplemented with 5% sheep blood. The agar surfaces were allowed to dry, and antimicrobial disks were applied on the plates. The plates were incubated at 37 °C for 48 h in a microaerophilic atmosphere. The inhibition zones were measured to the nearest millimeter using a graduated ruler [13]. *Staphylococcus aureus* ATCC 12600, *Escherichia coli* ATCC 11775 and *Campylobacter jejuni* ATCC 33291 were used as control strains. The antibiotic discs and their concentrations consisted of chloramphenicol (C, 30 μg, Bayer, Wuppertal, Germany), cephalothin (CF, 30 μg, Polfa, Tarchomin, Poland), rifampicin (RA, 30 μg), vancomycin (VA, 30 μg), ceftriaxone (CRO, 30 μg), trimethoprim (TMP, 30 μg), nalidixic acid (NA, 30 μg), clindamycin (DA, 15 μg), erythromycin (E, 15 μg), ciprofloxacin (CP, 15 μg), gentamicin (CN, 10 μg), amikacin (AK, 30 μg), tetracycline (TE, 30 μg), oxytetracycline (T, 30 μg), cefazolin (CZ, 30 μg), ampicillin (AM, 10 μg), kanamycin (K, 30 μg), enrofloxacin (ENR, 5 μg) which were obtained from Paramedical, Italia. Since there is no recommendation of breakpoint values for arcobacters, Clinical and Laboratory Standards Institute (CLSI) guidelines M45-A (for campylobacters) was used for erythromycin, tetracycline and ciprofloxacin [19], and CLSI M100–S20 (for Enterobacteriaceae) for all other antibiotics [20].

2.5. Statistical analysis

The results of the study were analyzed with the SPSS software version 16.1 (SPSS Inc., Chicago, IL, USA). The Pearson chi-square and Fisher's exact two-tailed tests were used to assess the association between the different isolation rates and type of animals and to determine the proportions of isolates resistant to different antimicrobial agents. *P* value less than 0.05 was considered statistically significant.

3. Results

Arcobacter spp. were isolated from 16 out of 200 (8%) cattle fecal samples. With PCR, 24 of 200 (12%) samples from cattle were positive for Arcobacters. Among 108 sheep fecal samples, 10.1% (11/108) and 18.5% (20/108) *Arcobacter* were isolated using culture and PCR, respectively. The predominant species was *A. butzleri* in

Table 1
Nucleotide sequences used as primers in the PCR reaction for identification of *Arcobacter* genus and *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* species.

Name of primer	Sequence (5′ to 3′)	Target gene	Annealing temperature	Product size (bp)	Reference
Arc 1 Arc 2	AGAACGGGTTATAGCTTGCTAT GATACAATACAGGCTAATCTCT	*16SrRNA*	44	181	[18]
Butz Arco	CCTGGACTTGACATAGTAAGAATGA CGTATTCACCGTAGCATAGC	*16SrRNA*	56	401	[17]
GyrasF GyrasR	AGAACATCACTAAATGAGTTCTCT CCAACAATATTTCCAGTYTTTGGT	Gyrase A	58	395	[18]
SkiR ArcoF	TCAGGATACCATTAAAGTTATTGATG GCYAGAGGAAGAGAAATCAA	23S rRNA	58	198	[18]

Table 2
Prevalence of *Arcobacter* species in cattle and sheep fecal samples.

Animal source	Number of samples	Positive for culture method (%)	Positive for genus specific PCR (%)	PCR results (%)			
				A. butzleri	*A. cryaerophilus*	*A. skirrowii*	*A. butzleri* + *A. cryaerophilus*
Cattle	200	16(8)	24(12)	14(58.3)	4(16.6)	2(8.3)	4(16.6)
Sheep	108	11(10.1)	20(18.5)	11(55)	2(10)	0(0)	7(35)
Total	308	27(8.7)	44(14.2)	25(56.8)	6(13.6)	2(4.5)	11(25)

Table 3
Percentage of *Arcobacter* isolates resistant to various antimicrobials.

Antimicrobial agent	Cattle		Sheep	
	A. butzleri (*N* = 13) (%)	*A. cryaerophilus* (*N* = 3) (%)	*A. butzleri* (*N* = 9) (%)	*A. cryaerophilus* (*N* = 2) (%)
Rifampicin	13(100)	3(100)	9(100)	2(100)
Vancomycin	13(100)	3(100)	9(100)	2(100)
Ceftriaxone	13(100)	3(100)	9(100)	2(100)
Trimethoprim	13(100)	3(100)	9(100)	2(100)
Nalidixic acid	6(46.1)	0	5(55.5)	0(0)
Clindamycin	11(84.1)	3(100)	9(100)	2(100)
Erythromycin	0(0)	0(0)	0(0)	0(0)
Ciprofloxacin	0(0)	0(0)	0(0)	0(0)
Gentamicin	0(0)	0(0)	1(11.1)	0(0)
Amikacin	0(0)	0(0)	1(11.1)	0(0)
Tetracycline	0(0)	0(0)	0(0)	0(0)
Oxytetracycline	0(0)	0(0)	0(0)	0(0)
Cefazolin	12(92.3)	3(100)	9(100)	2(100)
Ampicillin	11(84.1)	3(100)	8(88.8)	1(50)
Kanamycin	0(0)	0(0)	0(0)	0(0)
Chloramphenicol	5(38.4)	1(33.3)	4(44.4)	0(0)
Cephalothin	13(100)	3(100)	9(100)	2(100)
Enrofloxacin	1(7.6)	0(0)	0(0)	0(0)

cattle and sheep with 14 (58.3%) and 11 (55%), respectively. In addition, co-colonization of *A. butzleri* and *A. cryaerophilus* occurred in 25% of the positive samples as detected by species-specific PCR. The isolation rates of *A. butzleri* and *A. cryaerophilus* were not statistically different between cattle and sheep ($P > 0.05$). *A. skirrowii* was not detected in any of the sheep fecal samples. The occurrence rates of *Arcobacter* spp. isolated from the cattle and sheep using culture and PCR methods are shown in Table 2. All of the 27 *Arcobacter* isolates were resistant to one or more antimicrobial agents. The antimicrobial susceptibility of the isolates is shown in Table 3. All isolates of *A. butzleri* and *A. cryaerophilus* were found to be resistant to rifampicin, vancomycin, ceftriaxone, trimethoprim and cephalothin, and susceptible to tetracycline, oxytetracycline, erythromycin, ciprofloxacin, kanamycin, amikacin (except one isolate of *A. butzleri* from sheep), gentamicin (except one isolate of *A. butzleri* from sheep) and enrofloxacin (except one isolate of *A. butzleri* from cattle). Resistance of *A. butzleri* to nalidixic acid and chloramphenicol was significantly ($P < 0.05$) higher than *A. cryaerophilus*.

4. Discussion

Arcobacters have been classified as emerging foodborne pathogens in 2002 by the International Commission on Microbiological Specifications for Foods (ICMSF) [21]. The presence of arcobacters in feces of clinically healthy cattle in this study is consistent with previous studies that reported 9.5% [22] and 11% [23]. Van Driessche et al. [4] found high (39%) prevalence of *Arcobacter* in bovine fecal samples in Belgium. In addition, Vilar et al. [24] observed high occurrence (41.7%) of *Arcobacter* spp. in fecal samples of cattle in Spain. According to these studies, the prevalence of *Arcobacter* in pigs is higher than cattle and pigs can act as transmission agent for other species. The reported higher incidence of *Arcobacter* spp. in cattle [4,24] could be due to breeding of cows with pigs on the same farms. De Smet et al. [25] showed that small ruminants can play an effective role as a carrier of *Arcobacter*, on small and medium farms. Other studies showed that small ruminants can excrete *Arcobacter* in their feces [4,25,26]. Moreover, in this study *A. butzleri* was the most frequent species isolated from healthy cattle and sheep followed by *A. cryaerophilus* and *A. skirrowii* that were similar with other studies [4,22]. Ongor et al. [22] showed that only five percent of cows were colonized by *A. skirrowii*. In the present study, *A. skirrowii* was not isolated by the culture method; however, it was detected in two cattle samples using PCR. *Arcobacter* spp. were better detected by PCR (14.28%) than culture method (8.7%), it was also reported by Ferreira et al. [3]. Simultaneous presence or co-colonization of *Arcobacter* species was observed in 25% of positive samples. This phenomenon has been previously described by Van Driessche et al. [23] showing the existence of two or more species of *Arcobacter* in 26% of positive fecal samples of cattle.

The extended use of antibiotics can lead to development and spread of antibiotic resistant bacteria in human and animals [27]. In the present study, all *Arcobacter* isolates were resistant to rifampicin, vancomycin, ceftriaxone, trimethoprim and cephalothin, which may constitute an intrinsic resistance, as for vancomycin, or it may be acquired by prolonged antibiotic exposure, as is the case of quinolones [13]. In addition, a high level of resistance to clindamycin (92.59%), cefazolin (96.29%) and ampicillin (85.18%) was found in *Arcobacter* species. Fera et al. [28] had also reported high level of resistance to clindamycin in *A. butzleri* isolated from humans and animals. Ampicillin resistance rate of 97.7% was reported in *A. butzleri* isolates recovered from poultry and environment of a Portuguese slaughterhouse [13], which is comparable with the resistance observed in the present study (85.18%). Chloramphenicol was reported to be very active against *A. butzleri* in an earlier study [29]; however, 40.9% of *A. butzleri* and 20% *A. cryaerophilus* in this study were found to be resistant to this antibiotic. There are differences among studies regarding chloramphenicol susceptibility [3]. Otth et al. [30] suggested that these differences could be caused by local differences on the use of this antibiotic in livestock rearing. Moreover, Atabay

and Aydin [29] found that all strains of *A. butzleri* were susceptible to nalidixic acid, whereas a high prevalence of isolates resistant to nalidixic acid was observed in this study. The incidence of resistance against nalidixic acid in *A. butzleri* was higher compared to *A. cryaerophilus* ($P < 0.05$). Vandenberg et al. [31] suggested that fluoroquinolones could be used for treatment of severe *Arcobacter* enteritis. In another study, Son et al. [32] proposed that tetracycline along with aminoglycosides are suitable antibiotics for treatment of *Arcobacter* spp. The current study also demonstrated that all *Arcobacter* isolates were almost susceptible to amikacin, gentamicin, erythromycin, ciprofloxacin, enrofloxacin, tetracycline and oxytetracycline. Therefore, disease(s) caused by *Arcobacter* species can be treated with these antibiotics; however, the existence of resistant strains should be born in mind, as some isolates were resistant to some of those antibiotics (see Table 3 for details). This suggests that tetracycline may be useful for the treatment of human *Arcobacter* infections along with aminoglycosides.

In conclusion, the study indicated that healthy food animals such as cattle and sheep can play a significant role in the contamination of the environment and human food chain by *Arcobacter* spp. and are potential sources for carcass contamination during slaughter. Hence, in order to reduce fecal contamination of meat with *Arcobacter*, it is necessary to apply good hygienic standards and food safety assurance programs in the entire slaughtering process. Further epidemiological studies are needed in various areas of developing countries to ascertain the prevalence of *Arcobacter* infections. The antibiotic susceptibility tests showed that *Arcobacter* spp. are susceptible to aminoglycosides, tetracycline, erythromycin and ciprofloxacin. The presence of acquired resistance to erythromycin and ciprofloxacin among *Arcobacter* isolates is a matter of concern; because these antimicrobials are generally prescribe as first-line drugs for treatment of *Campylobacteraceae* infections in humans and that tetracycline or aminoglycosides may be useful for treatment of *Arcobacter* infections.

Acknowledgments

The researchers would like to thank the Research Council of Shiraz University and School of Veterinary Medicine, Shiraz University for financial and technical support of this study and to thank Dr. Sina Faghihzadeh Gorji for his review and kind help.

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