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Original article

PREVALENCE, VIRULENCE GENES AND ANTIMICROBIAL RESISTANCE OF *ARCOBACTER* ISOLATES FROM ANIMAL MEAT IN IRAN

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Summary

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Arcobacter spp. are food-borne and zoonotic entero-pathogens. Obtaining information in relation to antimicrobial resistance helps us for utilisation of an appropriate agent for the treatment of *Arcobacter* infections. This study aimed to investigate the prevalence, antimicrobial resistance and virulence factors in animal raw meat in Iran. The samples were collected from cattle (n=80), sheep (n=80), goats (n=80), camels (n=80), and buffaloes (n=60) from Khuzestan (n=110), Isfahan (n=80), Gilan (n=110) and Chaharmahal and Bakhtiari (n=80) provinces. *Arcobacter* isolates of meat samples were isolated, investigated by PCR method. The antibiotic resistance was also investigated. All isolates were screened for 6 virulence genes: *cadF*, *ciaB*, *cj1349*, *Mvin*, *pldA* and *tlyA* by PCR assays. The results showed that the prevalence of *Arcobacter* species had no significant difference among provinces and animals (P>0.05), so that positive samples were 1.25%, 1.25%, and 0.9% in Isfafhan, Chaharmahal and Bakhtiari, and Gilan, respectively. Virulence genes were observed for *A. butzleri* species (n=3, 100%). The results showed that *Arcobacter* spp. were resistant to streptomycin (100%), tetracycline (100%) and vancomycin (100%), but were susceptible to azithromycin (33.33%). In sum, the different regions of the Iran had a relative incidence of 1% for *Arcobacter* spp. The species showed a resistance of 100% for streptomycin, tetracycline and vancomycin. These findings could help to identify *Arcobacter* spp. and select the best agent against infection in case of *Arcobacter* infection in animals.

Key words: animals, antimicrobial resistance, *Arcobacter* species, meat, prevalence, virulence genes

INTRODUCTION

Arcobacters are food-borne and zoonotic entero-pathogens (Çelik & Ikiz, 2019). The *Arcobacter* genus belongs to *Campylobacteraceae* family, class Epsilonpro-

teobacteria of the phylum Proteobacteria (Fanelli *et al.*, 2019) and are most important pathogenic bacteria (Elmai & Can, 2017). Arcobacters are aerotolerant Gram-negative bacteria that grow in aerobic and microaerophilic conditions (mainly at 30 ^{o}C). The most known species are *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. nitrofigilis*, *A. cibarius* and *A. halophilus* (Van den Abeele *et al.*, 2014; Fernandez *et al.*, 2015). *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* cause infection, bacteraemia, endocarditis, peritonitis and diarrhoea (Elmai & Can, 2017). It was estimated that *A. butzleri* infections are mainly transmitted in humans by water routes (De Smet *et al.*, 2011). Abattoirs are potential sources for spreading disease (Collado *et al.*, 2010). Arcobacters are commonly isolated from healthy cattle, sheep and pigs (Ferreira *et al.*, 2016). Contaminations induced by *Arcobacter* species occur commonly during slaughter process (Van Driessche *et al.*, 2003). Increased trade of meat products between developing and industrialised countries increases the risk of the animal-associated pathogens in all over world (Dekker *et al.*, 2019) which is a major challenge in countries without surveillance systems and/or sites where pathogens can be introduced.

Polymerase chain reaction (PCR)-based methods are the commonest and most rapid methods for detection of the *Arcobacter* spp. from food and other samples (de Boer *et al.*, 2013). *Arcobacter* infections do not need drugs for treatment, but some agents, such as erythromycin, fluoroquinolones and ciprofloxacin were used for the treatment of infections (Skirrow, 2000). The utilisation of antibiotics has faced limitiations due to their antimicrobial resistance. It was reported that *A. butzleri* was more resistant than both *A. cryophilus* and *A. skirrowi* (Kabeya *et al.*, 2004; Abay *et al.*, 2012). Kabeya *et al.* (2004) showed that all *Arcobacter* strains were susceptible to ampicillin. Studies have reported susceptibility of *Arcobacter* strains to aminoglycosides and tetracycline (Collado & Figueras 2011; Abay *et al.*, 2012). Another study showed that the incidence of *Arcobacter* spp. was 27% in poultry meat (Lehmann *et al.*, 2015). Tabatabaei *et al.* (2014) investigated six virulence genes (*cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA*) in *Arcobacter* spp. and reported values of 87%, 45% and 60%, 90% and 80%, 33% and 13%, and 38% and 40% for *cadF*, *ciaB*, *cj1349*, *mviNin*, *pldA* and *tlyA* genes, respectively. Shirzad Aski *et al.* (2016) showed a occurrence of 9% (27/308) *Arcobacter* spp. in cattle and sheep from Southern Iran by cultural isolation and 14% (44/308) by PCR. The predominant species was *A. butzleri* in both cattle (58%) and sheep (55%) hence it was concluded that cattle and sheep were significant intestinal carriers for *Arcobacter* spp.

Virulence factors affecting the pathogenicity of these microorganisms are not completely known. Identification of virulence genes helps to know pathogenic nature of the isolated species of *Arcobacter*. Previous studies have not investigated the incidence, virulence genes and antimicrobial resistance of *Arcobacter* isolates from animals meat. With regard to *Arcobacter* spp., their contamination rate in animal meat, and providing an effective strategies for alleviation of the both the infections induced by *Arcobacter* spp.in humans and animals, this study investigated the rate of *Arcobacter* spp. contamination of animal meat. This study also investigated the antibiotic resistance profiles for *Arcobacter* spp. that can be used for developing a database for clinical

treatment of *Arcobacter* during its incidence in food products.

MATERIALS AND METHODS

Ethics approval

The study was performed in accordance with Ethical committe of Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Samples

The samples were obtained from cattle (n=80), sheep (n=80), goats (n=80), camels (n=80), and buffaloes (n=60) meat from January to December of 2018. The samples were collected from Khuzestan (n=110), Isfahan (n=80), Gilan (n=110) and Chaharmahal and Bakhtiari (n=80) provinces. The samples were immediately transported into a cool box and transferred to Food Quality control laboratory of Islamic Azad University, Shahrekord Branch. The meat samples (50 g) were randomly collected from different parts of animal carcasses at slaughterhouse (10 samples from per animal).

Isolation and identification of Arcobacter species

Arcobacter spp. was isolated as reported by Maruyama *et al.* (2001). Under sterile conditions, an amount of 5 g homogenised meat sample was transferred into 45 mL *Arcobacter* Selective Broth (ASB, Himidia, India) containing CAT selective supplement and incubated for 4 days at 25 ^{o}C. The samples were linearly cultured in *Arcobacter* specified media (ASB, Himidia, India) and the plates were then cultured for 4 days in 25°C. Typic colonies were selected in the plates, cultured in Brucella Agar media under same condition and assessed for phenotypic characteristics, catalase activity and nitrate reduction. To identify and differentiate *Arcobacter* species, DNA was extracted from meat samples by using DNA kit (Cinnagen Company, Tehran, Iran). The used primers were as followed: 1) *Arcobacter*): TTCGCTTGCGCTGCATCAT; 2) *A. butzleri*: AGCGTTCTATTCAGCGTAGAA GATGT; 3) *A. cryaerophilus*): ACCGAA GCTTTAGATTCGAATTTATTCA and 4) *A. skirrowii*: CGAGGTCACGGATG GAAGTG.

Amplification was conducted in a thermal cycler (Master Cycle Gradient, Eppendrof, Germany) as followed: initial denaturation at 94 ^{o}C for 10 min, followed by 30 amplification cycles consisting of denaturation for 30 s at 94 ^{o}C, annealing for 1 min at 64 ^{o}C and elongation for 1 min at 72 ^{o}C. The final elongation was performed at 72 ^{o}C for 7 min. The quality and quantity of extracted DNA was determined by measurement of the concentration. Purity was investigated by a UV spectrophotometer (NanoDrop™ 1000, Thermo Fisher Scientific,Waltham, MA, USA). Absorption was measured at 260 nm (A260) and 280 nm (A280). DNA purities were calculated through A260/ A280 ratios. Samples containing A260/ A280 ratios of 1.7–2.0 were considered as pure samples, free from protein and other contamination. Test method and thermal programme were as reported by Son *et al.* (2007). Agarose gel 1.5% was used to trace the products. DNA templates from reference strains were used as positive controls, and DNA was replaced with sterile distilled water in negative controls.

Antibiotic resistance

Antimicrobial susceptibility test was done by the Kirby–Bauer disc diffusion on by a Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084) supple-

Table 1. Primer pairs for tracing *Arcobacter* genes isolated from samples

Primer	Virulence factor	Primers	Amplicon size (bp)
cadF	Fibronectin protein	TTACTCCTACACCGTAGT AAACTATGCTAACGCTGGTT	283
ciaB	Invasive antigen	TGGGCAGATGTGGATAGAGCTTGGA TAGTGCTGGTCGTCCCACATAAAG	284
cj1349	Fibronectin protein	CCAGAAATCACTGGCTTTTGAG GGGCATAAGTTAGATGAGGTTCC	659
MviN	Virulence factor	TGCACTTGTTGCAAAACGGTG TGCTGATGGAGCTTTTACGCAAGC	294
pldA	Phospholipase A	TTGACGAGACAATAAGTGCAGC CGTCTTTATTTTGCTTTCAGGGA	293
tlyA	Haemolysin	CAAAGTCGAAACAAAGCGACTG TCCACCAGTGCTACTTCCTATA	230

mented with 5% defibrinated sheep blood as reported by Rahimi (2014). *Arcobacter* isolates of meat samples were investigated for antibiotic resistance for kanamycin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 µg), streptomycin (30 µg), tetracycline (15 µg), methicillin (30 µg), vancomycin (30 µg), azithromycin (10 µg), and nalidixic acid (30 µg) by the disc method as previously reported (Rahimi, 2014). *Staphylococcus aureus* (*S. aureus* ATCC 25923), and *Escherichia coli* (*E. coli* ATCC 25922) were used as quality control organisms in antimicrobial susceptibility determination.

Virulence genes

All isolates were screened for 6 virulence genes, including *cadF*, *ciaB*, *cj1349*, *Mvin*, *pldA and tlyA* by PCR assays. To identify the virulence genes, all strains isolated in this study underwent PCR using the primers listed in Table 1.

Data analysis

The data were analysed by SPSS software and by Chi-square and Fisher test. The data were reported as number and frequency (%).

RESULTS

Prevalence

The results for incidence of *Arcobacter* species in different provinces are shown in Table 2. The results showed that positive samples were 1.25% (1/80), 1.25% (1/80) and 0.9% (1/110) in Isfafhan, Chaharmahal and Bakhtiari, and Gilan, respectively. The lowest incidence was observed in Khuzestan province. Highest and lowest rates for the incidence were observed for cattle (2/80, 2.50%) and buffaloes (1/60, 1.66%) respectively (Table 3). In all the positive samples, *A.butzleri* was found out (Fig. 1).

Antimicrobial resistance

The results for antimicrobial resistance are shown in Table 4. *Arcobacter* isolates were resistant to streptomycin (3/3, 100.00%), tetracycline (3/3, 100.00%) and vancomycin (3/3, 100.00%), but susceptible to azithromycin (1/3, 33.33%). *Arcobacter* had a medium susceptibility to

clindamycin (2/3, 66.66%), erythromycin (2/3, 66.66%), and nalidixic acid.

Virulence genes

The results for 6 virulence genes including *cadF*, *ciaB*, *cj1349*, *Mvin*, *pldA* and *tlyA* are shown in Table 5. All *A. butzleri* isolates were positive for *cadF*, *ciaB*, *cj1349*, *Mvin*, *pldA* and *tlyA*.

DISCUSSION

Increased prevalence of *Arcobacter* spp. in food, and its antimicrobial resistance have pushed researchers to conduct studies on these bacteria for food safety. Their incidence is increasing in water and food in some countries. Previous studies have investigated the incidence in different regions, and/or investigated antimicrobial resistance. A few studies investigated the effects of virulence genes. No study has investigated the incidence, virulence genes and antimicrobial resistance of *Arcobacter* isolates from animal meat. The information about the incidence, virulence genes and antimicrobial resistance can help to identify the best antibiotic. On the other hand, virulence factors affecting the pathogenicity of these microorganisms are not completely known. Identification of virulence genes would reveal the pathogenic nature of the isolated *Arcobacter* species, therefore the need to conduct a study that investigates the incidence, virulence genes and antimicrobial resistance of *Arcobacter* isolates from animal meat in Iran. In sum, the results reported only 3 positive samples observed in Isfafhan (n=1), Chaharmahal and Bakhtiari (n=1), and Gilan (n=1). Two positive samples from cattle and 1 positive sample from buffalo were observed, e.g. different animals did not show significant difference for infection rates with *Arcobacter*. All the samples were positive for *A.butzleri.*

Table 2. The incidence of *Arcobacter* spp. in different provinces

Samples	Number	Positive samples	*Arcobacter* species		
			A. butzleri	*A. cryaerophilus*	*A. skirrowii*
Isfahan	80	1 (1.25%)	1 (100.00%)	–	–
Chaharmahal and Bakhtiari	80	1 (1.25%)	1 (100.00%)	–	–
Khuzestan	110	–	–	–	–
Gilan	110	1 (0.90%)	1 (100.00%)	–	–

Table 3. The incidence of *Arcobacter* spp. in meat of different animals

Samples	Number	Positive samples	*Arcobacter* species		
			A. butzleri	*A. cryaerophilus*	*A. skirrowii*
Cattle	80	2 (2.50%)	2 (100.00%)	–	–
Sheep	80	–	–	–	–
Goat	80	–	–	–	–
Camel	80	–	–	–	–
Buffalo	60	1 (1.66%)	1 (100.00%)	–	–

In contrast to our findings, Shirzad Aski *et al.* (2016) investigated the occurrence of *Arcobacter* spp. in cattle and sheep in Southern Iran and reported 9% and 14% positive samples for cattle and sheep. Similarly, they reported samples positive for *A. butzleri* in both cattle (58%) and sheep (55%). In this study, difference among animals was not observed, but predominance in cattle (2 cases) may be attributed to the fact that beef meat provides an appropriate environment for *Arcobacter* growth. Incidence rate of *Arcobacter* spp. from healthy cattle is different in the different regions that shows presence without any clinical manifestation and also cattle can act as a reservoir. In the current study, incidence rate was low that may be attributed to geographical condition and animal species that cause the bacteria could not significantly grow. Merga *et al.* (2011, 2013) reported the incidence of 43% and 40% from faeces of cattle and sheep in theUnited Kingdom. A difference between our findings and others could be attributed to sampling types (meat vs feces). The results showed that *A. butzleri*

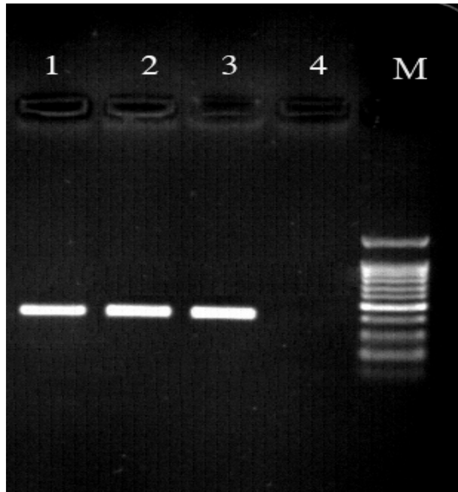

Fig. 1. Agarose gel electrophoresis of *A. butzleri.* Lanes 1 and 2: isolates positive for *A. butzleri*, lane 3: positive control, lane 4: negative control; lane M: ladder size.

Table 4. Antibiotic resistance of *Arcobacter* species isolated from animal meat

Antibiotics	*A. butzleri* (n=3)	*A. cryaerophilus (n=0)*	*A. skirrowii (n=0)*
Kanamycin	–	–	–
Gentamicin	–	–	–
Chloramphenicol	–	–	–
Clindamycin	2 (66.66%)	–	–
Erythromycin	2 (66.66%)	–	–
Streptomycin	3 (100.00%)	–	–
Tetracycline	3 (100.00%)	–	–
Meticillin	2 (66.66%)	–	–
Vancomycin	3 (100.00%)	–	–
Azithromycin	1 (33.33%)	–	–
Nalidixic acid	2 (66.66%)	–	–

Table 5. Virulence genes in *Arcobacter* spp. isolated from animal meat

Species	*cadF*	*ciaB*	*cj1349*	*Mvin*	*pldA*	*tlyA*
A. butzleri (n=3)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)
A. cryaerophilus (n=0)	–	–	–	–	–	–
A. skirrowii (n=0)	–	–	–	–	–	–

was the organism isolated from positive samples. *A.butzleri* is the commonest organism isolated in dairy farms compared to other *Arcobacter* species that is due to the ability of this organism to survive in different environments (Giacometti *et al.*, 2015). Badilla-Ramırez *et al.* (2016) reported that *A. butzleri* could grow at 4 and 10 ^{o}C and some meats may provide conditions for its growth. It was also reported that *A. butzleri* is a strong bacterium that survives at different storage temperatures, posing potential health risk for poultry meat consumers (Ramees *et al.* 2017).

Antimicrobial resistance must be investigated, because it helps to use the best antibiotic as first-line drug for the treatment of *Arcobacter* infection (Houf *et al.*, 2004; Vandenberg *et al.*, 2006). All isolates showed resistance to streptomycin, tetracycline and vancomycin, but were susceptible to azithromycin. *Arcobacter* spp. had a medium susceptibility to clindamycin, erythromycin, and naidixic acid. Similarly, Yesilmen *et al.* (2014) reported that the acquired resistance to tetracycline among the food-borne isolates may raise concern for the treatment of *Arcobacter* infections in the animals, because it is be commonly used for the treatment of infections induced by *Arcobacter* species. Other studies have reported high susceptibility of *Arcobacter* species to tetracycline (Atabay & Aydin, 2001; Kabeya *et al.*, 2004; Zacharow *et al.*, 2015). Shah *et al.* (2013) reported that *Arcobacter* species wee susceptible to gentamicin. It can be concluded that *A. butzleri* isolated from animal meat was the most susceptible to azithromycin.

The results showed that all the samples were positive for *cadF*, *ciaB*, *cj1349*, *Mvin*, *pldA* and *tlyA*. The genes perform different activities, for example, *cadF* and *cj1349* genes code outer membrane proteins and facilitate intestinal epithelial cell to cell contact through adhering to fibronectin (Douidah *et al.*, 2012). The *ciaB* gene is involved in invasion of host cell and *pldA* gene codes for outer membrane phospholipase A that hydrolyses acyl ester bonds, but *tlyA* gene is a haemolysin gene (Douidah *et al.*, 2012). In a study, Tabatabaei *et al.* (2014) investigated six virulence genes in *Arcobacter* spp. in Southern Iran and reported six genes present in all the *A. butzleri* isolates. Obtaining information in relation to virulence genes helps to identify the pathogenic nature of the isolated species of *Arcobacter*. Despite to adhere and invade *A. butzleri* into different lines, no correlation was reported between *A. butzleri* virulence genes and adhesive and invasive phenotypes (Karadas *et al.*, 2013; Levican *et al.*, 2013). Similarly, several studies have reported six virulence genes in *Arcobacter* spp. isolates (Karadas *et al.*, 2013; Lehmann *et al.*, 2015). Draft genome of *Arcobacter* helps to know their roles in exploring the various genes and in virulence and pathogenicity (Adam *et al.*, 2014a, b, c). Whiteduck-Leveillee *et al.* (2016) showed that the occurence of virulence genes *ciaB*, *mviN*, *tlyA* and pldA was significantly higher in *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.

CONCLUSION

In sum, the study did not demonstrate significant difference among the incidence of *Arcobacter* spp. in animals and provinces. The incidence was insignificant (1%) and was associated to *A. butzleri*. *A. butzleri* was resistant to streptomycin, tetracycline and vancomycin, but susceptible to azithromycin. The identification of virluence factors and antibacterial resistance help to identify and schedule the

control of *Arcobacter*. Since high levels of contamination with *Arcobacter* spp. can occur in slaughterhouses, the maintenance of slaughter hygiene and regular microbiological monitoring of carcasses are essential for decreasing the risk of contamination especially when animal species with lower incidence levels are slaughtered on the same slaughtering line.

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