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RESEARCH ARTICLE

ANTIBIOTIC SUSCEPTIBILITY AND PRESENCE OF VIRULENCE GENES IN *ARCOBACTER BUTZLERI* STRAINS ISOLATED FROM POULTRY, COSTA RICA

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ABSTRACT

Arcobacter butzleri, is an emerging potential foodborne zoonotic pathogenthat has been classified as a serious hazard for human health by the International Commission on Microbiological Specifications for Foods (ICMSF). In Costa Rica, there are several reports about *Arcobacter*, nevertheless there is scarce information about the adhesion, invasion and pathogenic features of this bacteria, or the virulence genes associated. Also, information regarding its susceptibility to antibiotics is controversial due to a lack of a standardized technique and interpretation guidelines. The aim of this work was to determine the antibiotic susceptibility and presence and frequency of several virulence genes in 26 strains of *A. butzleri* previously isolated in Costa Rica. There was an 87,5% resistance to nalidixic acid, and 8,3% resistance to gentamicin and ampicillin. Also, one strain was multiresistant showing simultaneous resistance to ampicillin, gentamicin and nalidixic acid. Gene *cadF* was the most prevalent one, being present in 10 of the 26 isolates analyzed, followed by genes *ciaB* and *irgA* that showed a 23% frequency. Genes *mviN* and *tlyA* were the less prevalent ones, being isolated just from one strain each. Eight isolates did not present any of the genes analyzed. Further research shall be done in order to establish a standardized technique for the determination of antibiotic susceptibility and interpretation guidelines for this bacteria as well as in the correlation among the presence of virulence genes and its expression.

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INTRODUCTION

Food borne infections have acquired growingimportance in the last years, due to an increase in thenumber of outbreaks throughout the world and to the emergence of new agents (Lehmann, 2015). This is the case of *Arcobacter butzleri,* an emerging potential foodborne zoonotic pathogen (Houf, 2001). Although this is a little studied bacteria, the International Commission on Microbiological Specifications for Foods (ICMSF) has classified it as a serious hazard for human (Houf, 2001). Genus *Arcobacter* was proposed by Vandamme *et al* in 1991 (Vandamme, 1991), nowadays, 25 different species have been reported (Levican, 2013A; Ramees, 2017). From these*, A. butzleri, A. cryaerophilus, A. skirrowii* and *A. thereius*are considered as emergent food borne pathogens (McGregor and Wright, 2015; Ramees, 2017). The principal transmission route for humans is through the consumption and handling of raw or undercooked animal products, including meat, milk or seafood (Atabay and Aydin, 2001; Shah, 2011).Poultry meat shows the highest prevalence of this bacteria (40-100%), followed by pork, seafood (7-54%) and bovine meat (2,2-38%) (Phillips, 2001A; Shah, 2011). There are several reports of the isolation of *Arcobacter* from abattoirs; also its persistence has been recorded even after disinfection procedures, so these facilities may also act as potential source of spead (Collado, 2010). Among all arcobacters, A. *butzleri* is the most frequently isolated one, from clinical, environmental and food samples (Atabay, 2003; Merga, 2011; Amare, 2011). It has been associated with human disease, including enteritis, severe diarrhea, bacteremia and septicemia (Van den Abeele, 2015; Webb, 2016). In Costa Rica, there are several reports about *Arcobacter*, almost all of them in poultry products but also in bovine meat (Córdoba, 2017). The ability of this group of agents to produce disease, such as human and animal enteritis, has been proved. Nevertheless, there is a limited knowledge about their pathogenesis and infecting dose (Lehner. 2005; Collado and Figueras, 2011).There is scarce information about the adhesion, invasion and pathogenic features of this bacteria,

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or the virulence genes associated to this genus.A better description of the virulence genes such as *cadF, ciaB, cj1349, hecA, hecB, irgA, mviN, pldA, tlyA and iroE* found in *A. butzleri* could be made after latest descriptions of the genome of this pathogen. Some of these virulence genes present strong homology with similar genes found in *Campylobacter jejuni* whose functionality is already known (Girbau, 2015). *Campylobacter* and *Arcobacter* are closely related microorganisms, associated to poultry and to the production of human disease (Vandamme, 1991), reason why there is a strong homology in the virulence genes they present. Same way, a high amount of genetic diversity and an increase in antibiotic resistance has been reported among *Arcobacter* isolates in different parts of the world (Bagakalote, 2014; Ferreira, 2016). Fluoroquinolones and ampicillin are first choice therapy for the treatment of *Arcobacter* clinical cases, nevertheless, increasing resistance rates have been reported by different authors (Van den Abeele, 2010). The aim of this work is to determine the antibiotic susceptibility of *A. butzleri* strains isolated in Costa Rica and to determine the presence and frequency of several virulence genes in order to define their potential risk as agents of foodborne diseaseand to help understand the pathogenesis and potential treatment associated to this bacteria (Merga, 2011).

MATERIAL AND METHODS

Samples: Twenty six *A. butzleri* strains, previously isolated and identified from poultry samples from Costa Rica, were evaluated for antibiotic susceptibility and the presence of different virulence genes.

Antibiotic susceptibility test: E-test methodwas used for determining antibiotic susceptibility. Mueller Hinton agar supplemented with 5% blood was used. Antibiotics tested included ampicillin, gentamicin, nalidixid acid, ciprofloxacin and levofloxacin. Control strains included *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Because there is no standard procedure for the antibacterial susceptibility testing for *Arcobarcter,*the breakpoints used for ciprofloxacin are the ones described for *Campylobacter* species according to EUCAST guidelines (EUCAST 2017) and for the other antibiotics the Clinical and Laboratory Standars Institutes guidelines (CLSI 2015) for *Campylobacter* were used as cut off concentrations (Table 1).

Identification of virulence genes

Virulence genes were identified using the protocol described by Douidah *et al.*(2010), that includes 9 different virulence genes(Douidah, 2010).Primers used are described in Table 2. The thermocycling program was: 94°C for 3 min; 94°C for 145 s, 56 °C for 45 s and 72 °C for 45 s (32 cycles); and 72 °C for 3 min. Analysis of PCR products was done by electrophoresis (60 V, 1.5 h) in 1.5% agarose gels (w/v) with Mass Ruler (100-1000bp) and GelRed staining.

RESULTS

Antibiotic susceptibility: The antibiotic resistance patterns from 24 different *A. butzleri* isolates are shown as minimal inhibitory concentration (MIC (µg /mL) on Table 3. It is important to point out that after freezing, two isolates could not be recovered again for the antibiotic susceptibility test. These results show that there was an87,5% resistance to nalidixic acid, 30% resistance to ciprofloxacin, 12,5% resistance to ampicillin and 8,3% resistance to gentamicin. Strains isolated were al susceptible to levofloxacin. Also, one strain was multiresistant showing resistance to ampicillin, gentamicin and nalidixic acid.

Presence of virulencegenes: Results obtained for virulence genes are exposed on Table 4.

Table 1. Standardized cut off values for the determination of resistance and susceptibility for *Campylobacter* (CLSI 2015)

Antibiótic	Cut off value (µg/mL)
Ampicillin	≥32
Ciprofloxacin	≥4
Gentamicin	≥16
Levofloxacin	≥8
Nalidix acid	≥32

Table 2. Sequence of nucleotides used as primers for the detection of virulence genes of *Arcobacter*

Primer	Target gen	Secuencia	Tamaño (bp)
cadF-F	cadF	TTACTCCTACACCGTAGT	283
cadF-R		AAACTATGCTAACGCTGGTT	
ciaB-F	ciaB	TGGGCAGATGTGGATAGAGCTTGGA	284
ciaB-R		TAGTGCTGGTCGTCCCACATAAAG	
cj1349-F	cj1349	CCAGAAATCACTGGCTTTTGAG	659
cj1349-R		GGGCATAAGTTAGATGAGGTTCC	
mviN-F	mviN	TGCACTTGTTGCAAAACGGTG	294
mviN-R		TGCTGATGGAGCTTTTACGCAAGC	
pldA-F	pldA	TTGACGAGACAATAAGTGCAGC	293
pldA-R		CGTCTTTATCTTTGCTTTCAGGGA	
tlyA-F	tlyA	CAAAGTCGAAACAAAGCGACTG	230
tlyA-R		TCCACCAGTGCTACTTCCTATA	
irgA-F	irgA	TGCAGAGGATACTTGGAGCGTAACT	437
irgA-R		GTATAACCCCATTGATGAGGAGCA	
hecA-F	hecA	GTGGAAGTACAACGATAGCAGGCTC	537
hecA-R		GTCTGTTTTAGTTGCTCTGCACTC	
hecB-F	hecB	CTAAACTCTACAAATCGTGC	528
hecB-R		CTTTTGAGTGTTGACCTC	

Traditional PCR amplification was performed in reaction mixture (50 µL) containing Tris-HCl buffer (pH7.4), 0.2 mMdNTPs, 1.5 mM $MgCl_2$, 2 µM each primer, 1.5 U/µL Taq polymerase (Oxoid) and 2 µL of template DNA.

Table 3. Antibiotic resistance patterns of 24 different *A. butzleri* isolates, MIC RANGENumber *A. butzleri* isolates with MIC

Antimicrobial	S	I	R	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	% R
Ampicillin	≤8	16	≥32	7	1	3	3	3	1	1				1	1		1	12,5
Ciprofloxacin	≤0,5		≥0,5	6		3	5	3	7									30
Levofloxacin	1		≥8	6	5	10	2	1										0
Nalidixic acid	≤16		≥32			1							2	6	4		11	87,5
Gentamicin	≤4	8	≥16	7	2	7	4	1	1						1		1	8,3

Table 4. Presence of different virulence genes on *A. butzleri* species isolated

Gene	*cadF*	*ciaB*	*cj1349*	*irgA*	*hecA*	*hecB*	*mviN*	*pldA*	*tlyA*
Number of strains expressing the virulence gene (%)	10/26 (38,5%)	6/26 (23%)	0/26 (0%)	6/26 (23%)	0/26 (0%)	5/26 (19,2%)	1/26 (3,8%)	3/26 (11,5%)	1/26 (3,8%)

Gene *cad F* was the more prevalent one, being present in 10 of the 26 isolates analyzed, followed by genes *ciaB* and *irgA* that showed a 23% frequency. Genes *mviN* and *tlyA* were the less prevalent ones, being isolated just from one strain each. One of the strains isolated presented the highest number of virulence genes, presenting 4 of the 9 genes analyzed at same time, followed by three strains that presented 3 genes at same time. Eight isolates did not present any of the genes analyzed.

DISCUSSION

Commonly, infections caused by *Arcobacter* turn to be auto limited, and there is no need to begin an antimicrobial therapy for its resolution. Nevertheless, if symptoms persist antimicrobials have to be used. The most common used ones are erythromycin and ciprofloxacin (Shirzard, 2016). There is very limited data regarding antimicrobial susceptibility of *Arcobacter* (Ramees, 2017) and the comparison of susceptibility results might be controversial due to a lack of a standardized technique and interpretation guidelines(Shirzard, 2016). Literature reports show divergent rates of resistance to these antibiotics, varying from very high resistance rates up to very low or complete absence of resistance (Sen, 2007), depending on the geographical area where the study was performed and the methodology used (Kayman, 2012). As shown on table 3, isolates tested in this work show no resistance to levofloxacin, nevertheless there is a 8.3% resistance to gentamicin, 12,5% to ampicillin, 30% to ciprofloxacin and a 87.5% to nalidixic acid. Kayman *et al.*also describe a 100% susceptibility for ciprofloxacin on isolates obtained at Turkey (2012) using same methodology as we did (Kayman, 2012), same way, Van den Abeele et al report a 72% susceptibility in Belgic (Van den Abeele, 2016), but Cordoba *et al*. reported a 100 resistance in Costa Rica but using the agar diffusion method and for isolates obtained from minced meatsamples (Cordoba, 2017). For levofloxacin results reported worldwide are also contradictory, there are studies that report 100% resistance(Córdoba, 2017)and studies that report a small resistance as Gonzalez *et al.* that report only 8% (González, 2017). Our resistance results, obtained using the same methodology as Gonzalez for this antibiotic is very similar. For nalidixic acid, we found a 87.5% resistance. Cordoba *et al*. also report a 100% resistance (Córdoba 2017), but Kayman et al reported just a 22% (Kayman, 2012). Resistance to quinolones and fluoroquinolones is due to a single point mutation in a determinant region for *gyr A* gene, a fact that explains the increase of resistance to these groups of antibiotics in a short time. Increases in the resistance of *Arcobacter* to fluoroquinolones are a concern, since these compounds are the first choice treatment for *Campylobacter* and *Arcobacte*r infections (Collado and Figueras, 2011). For both gentamicin and ampicillin, the resistance found in this work is 8.3 and 12,5% respectively, . Van den Abbele *et al* report 1% resistance for gentamicin (Van den Abeele, 2016), and Cordoba *et al.* (Córdoba, 2017) and Kayman *et al.* (Kayman, 2012) reported no resistance. For ampicillin, high resistance rates have been reported. Van den Abeele *et al*reported a 91% resistance (Van den Abeele, 2016) and Kayman*et al*. and Córdoba *et al*.a 100% resistance (Kayman, 2012; Córdoba, 2017). The extended ampicillin resistance shown by these bacteria may be due to the presence of a beta lactamase as it was pointed out by Otth *et al* (Otth, 2004). Multidrug resistance has also been reported by different authors, including Van den Abeele *et al.* (Van den Abeele, 2016) and Collado *et al* (Collado, 2010).

Virulence genes

There is scarce research regarding the virulence genes in *Arcobacter* (Otth, 2004). Ten putative genes have been recognized in *A. butzleri* ATCC 49616 genome, including the ones studied in this research and *iroE* (González, 2017). The isolates studied presented as most prevalent the genes *cadF, ciaB* and *irgA. The cadF*gene codes for outer membrane proteins that facilitates intestinal epithelial cell to cell contact by adhering fibronectin, *ciaB* is associated to invasion of host cell and *irgA* to the codification of enterobactic, an outer membrane receptor (Douidah, 2012). Different authors also report the presence of several virulence genes in *Arcobacter* strains. Karadas *et al.* (Karadas, 2013) and Lehmanmn *et al* (Lehmann, 2015) report the presence of six virulence genes including *ciaB, cadF, cj2345, irgA* and *hecA* also reported in this paper. These virulence gens were detected in strains isolated from meat, shellfish, sewage, feces from pigs, sheep and chickens, environmental sources, piggery effluent, etc (Levican, 2013B). Contrasting with our results, Whiteduc Leveillee *et al.* (Whiteduck, 2016A) reported a high prevalence of *ciaB, mviN, tlyA* and *pldA* genes, and a lower one for *hecA, hecB, irgA* and *cj1249*. Our results show a low prevalence or even abscenceof *cj1249, mviN* and *tlyA* genes. Isolates tested present resistance to different common use antibiotics, specially to nalidix acid. This resistance reveals that the potential infection with this bacteria might represent a real risk for human beings. Also, the presence of these virulence genes reveals the potential pathogenic nature of the isolates tested. Nevertheless further research shall be done in order to establish a correlation among gene presence and its expression. Also, more research has to be done in order to understand the pathogenicity of this bacteria.

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