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Virulence genotypes and antimicrobial susceptibility patterns of *Arcobacter butzleri* isolated from seafood and its environment

Srinu Rathlavath[a], Vandita Kohli[a], Asem Sanjit Singh[a], Manjusha Lekshmi[a], Gayatri Tripathi[b], Sanath Kumar[a], Binaya Bhusan Nayak[a,*]

[a] Post-Harvest Technology, ICAR - Central Institute of Fisheries Education (CIFE), Mumbai 400061, India
[b] Aquatic Environment and Health Management, ICAR- Central Institute of Fisheries Education (CIFE), Mumbai 400061, India

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ABSTRACT

Arcobacter butzleri is an emerging pathogen isolated from animals, food and the environment. In this study, 147 *A. butzleri* isolated from seafood and the coastal environment were tested for the presence of ten putative virulence genes (*cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB*, *irgA*, *iroE*) and antimicrobial susceptibilities. Majority of the isolates harbored *mviN* (100%), *cj1349* (97.2%), *ciaB* (95.9%), *tlyA* (91.8%), *pldA* (91.1%) and *cadF* (89.7%). Lower detection rates were observed for *hecA* (10.8%), *hecB* (19%), *iroE* (12.9%) and *irgA* (17.6%). Three *A. butzleri* isolates harbored all ten virulence genes. The occurrence of *cj1349*, *ciaB*, *pldA*, *tlyA and hecA* genes was significantly different ($P \leq 0.05$) among the isolates from different sources. All (100%) *A. butzleri* isolates were resistant to vancomycin, cephalothin, cefoxitin and sulphamethizole and susceptible to polymyxin-B, kanamycin, streptomycin, gentamicin, tetracycline and imipenem. Resistance to clinically important antibiotics such as cefotaxime (99.3%), ceftazidime (87.7%), nalidixic acid (70.7%), ampicillin (72.1%), ertapenem and amoxicillin-clavulanic acid (41.9%) was observed in *A. butzleri* from the environment. The isolates were highly susceptible to norfloxacin (97.9%) and colistin (97.2%), followed by ciprofloxacin (88.4%), meropenem (74.8%), chloramphenicol (72.7%) and erythromycin (69.3%). *A. butzleri* from different sources were not significantly different with respect to their antimicrobial susceptibility patterns. Multidrug resistance was observed in 66 (81.4%) isolates from fish, 29 (72.5%) isolates from shellfish and 17 (65.3%) isolates from coastal water. *A. butzleri* harboring virulence genes and resistance to multiple antibiotics found in seafood could be a potential health risk to seafood handlers and consumers. Continuous monitoring of seafood for potentially pathogenic *A. butzleri* is important to understand the evolution of antibiotic resistance in this emerging food pathogen and to determine the antimicrobial therapy regimen in the event of food-borne *A. butzleri* infections.

1. Introduction

The genus *Arcobacter*, previously known as the aero-tolerant *Campylobacter*, comprises of non-spore forming, motile, slender, slightly curved Gram-negative bacteria that differ from the closely related campylobacters by their ability to grow aerobically and at temperatures from 15 to 30 °C (Vandamme et al., 1991). Currently, the genus *Arcobacter* has 25 recognized species (Diéguez et al., 2017). A few *Arcobacter* species are known to cause infections in both animals and humans and are considered enteropathogenic and zoonotic (Ho et al., 2006). However, only three species namely *Arcobacter butzleri*, *Arcobacter skirrowii* and *Arcobacter cryaerophilus* are reported to be pathogenic to humans (Vandenberg et al., 2004). In particular, *A. butzleri* has been linked with several cases of gastrointestinal disease in humans with diarrhoea being the main symptom (Collado and Figueras, 2011; Ferreira et al., 2014). The International Commission on Microbiological Specifications for Foods has classified *A. butzleri* as a serious hazard to human health (ICMSF, 2002). Contamination of foods of animal origin is assumed to occur during slaughter process through handling (Van Driessche et al., 2003) or from contaminated water used for cleaning. *A. butzleri* have been frequently found in fish, shell fish and water samples (Collado et al., 2009; Fera et al., 2004; Laishram et al., 2016), although the sources of this bacterium in seafood are not clearly understood.

Infections by *Arcobacter* spp. are normally self-limiting and do not require antimicrobial therapy, although in severe cases of *Arcobacter* enteritis antibiotic treatment may be advocated (Collado and Figueras, 2011). *Arcobacter* spp. are generally sensitive to fluoroquinolones, tetracyclines and aminoglycosides (Vandenberg et al., 2006), and hence these antibiotics are recommended as treatment options in cases of severe infections (Abay et al., 2012; Son et al., 2007; Vandenberg et al.,

* Corresponding author at: QC Laboratory, Post-Harvest Technology Department, Central Institute of Fisheries Education (CIFE), Mumbai 400061, India.
E-mail address: nayakbb@cife.edu.in (B.B. Nayak).

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2006). Several recent studies have reported the antimicrobial susceptibility patterns of arcobacters from environment, animal and human origin (Ferreira et al., 2016). Development of antibiotic resistance by pathogenic bacteria is a major challenge in the contemporary world. Among various reasons for the development of antibiotic resistance, exposure to sublethal levels of antibiotics and acquisition of resistance genes from other bacteria in the same environment are important (Bronzwaer et al., 2008; Van den Bogaard and Stobberingh, 2000).

Understanding the pathogenicity of *A. butzleri* for humans requires information on virulence factors. However, current knowledge of potential virulence factors and key mechanisms (e.g., adhesion, invasion, and cytotoxic capacity) of *A. butzleri* is still limited (Douidah et al., 2012; Karadas et al., 2013; Levican et al., 2013). The whole genome sequencing of *A. butzleri* RM4018 has helped to identify several putative virulence genes, some of which are homologous with *Campylobacter jejuni* virulence determinants (Miller et al., 2007). Several genes such as *cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB*, *irgA*, *IroE* in *A. butzleri* are considered important for the virulence of this pathogen, owing to their homology with virulence genes found in other pathogenic bacteria (Miller et al., 2007).

Most reports of food and water-borne illnesses by *A. butzleri* are associated with the consumption of foods of animal origin or of contaminated water (EFSA and ECDC, 2015). Considering the fact that *A. butzleri* could be isolated from meat, water and seafood in India, the possibility of this potential human pathogen acquiring antibiotic resistance exists. In particular, *A. butzleri* in seafood co-occur with other pathogenic bacteria which harbour multiple antibiotic resistance genotypes (Singh et al., 2016). The true disease burden due the occurrence of pathogenic and antibiotic resistant *A. butzleri* in foods of animal origin is unknown in India, since clinical reports on the incidences of *Arcobacter* infections are lacking. In this context, the aim of this study was to understand the pathogenic potentials of *A. butzleri* isolated from different sources such as fish, shellfish and coastal water samples and their antibacterial susceptibilities.

2. Materials and methods

2.1. Arcobacter butzleri isolates

One hundred-forty seven isolates of *A. butzleri* from fish (81), shellfish (40) and coastal water (26) were used in this study. The isolates were identified initially using a genus-specific PCR, followed by confirmation of the species (*A. butzleri*) using a multiplex-PCR and a 16SrRNA PCR-RFLP (Rathlavath et al., 2017). A reference strain of *A. butzleri* (ATCC 49616) was included as the positive control in all PCR assays.

2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of *A. butzleri* isolates was tested by disk diffusion technique according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). The antibiotics tested included amoxicillin-clavulanic acid (AMC, 30 μg), ampicillin (AMP, 10 μg), cefotaxime (CTX, 30 μg), cefoxitin (CX, 30 μg), ceftazidime (CAZ, 30 μg), cephalothin (CEP, 30 μg), chloramphenicol (C, 30 μg), ciprofloxacin (CIP, 5 μg), colistin (CL, 5 μg), ertapenem (ETP, 10 μg), erythromycin (E, 15 μg), gentamicin (GEN, 10 μg), imipenem (IMP, 10 μg), kanamycin (K, 30 μg), meropenem (MRP, 10 μg), nalidixic acid (NA, 30 μg), norfloxacin (NX, 10 μg), polymyxin-B (PB, 300 units), streptomycin (S, 10 μg), sulphamethizole (SM, 300 μg), tetracycline (TE, 30 μg) and vancomycin (VA, 30 μg) (Hi-Media, India). *A. butzleri* isolates were grown in Mueller Hinton (MH) broth under microaerophilic condition to an optical density of 0.5 and a 0.1 ml aliquot was spread plated on Muller Hinton agar. Antibiotic discs were dispensed on the inoculated plates and incubated at 30 °C under microaerophilic atmosphere (IR-water jacketed CO_2 incubator, NuAire, MN, USA). The zones of inhibition were recorded and interpreted according to CLSI guidelines (CLSI, 2015).

2.3. DNA preparation and PCR detection of Arcobacter butzleri VGs

Genomic DNA extraction of *A. butzleri* isolates was done using CTAB (cetylltrimethyl ammonium bromide) method (Ausubel et al., 1995). The presence of ten putative virulence genes (VGs) was determined by using PCR assays developed by Douidah et al. (2012) and Karadas et al. (2013). All PCRs were carried out in a final volume of 25 μl containing 100 ng of DNA template. The amplified products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide and photographed using a gel documentation system (Bio-Rad, Hercules, CA).

2.4. Statistical analysis

For statistical analysis, Kruskal-Wallis H test for k independent samples was performed to analyze the association of the ten virulence genes in *Arcobacter* isolates from different sources using SPSS statistical software (SPSS Inc., Chicago, IL, USA). *P*-value of ≤ 0.05 was considered statistically significant.

3. Results and discussion

3.1. Occurrence of putative virulence genes (VGs) in A. butzleri

Although seafood-borne *A. butzleri* infection has not been reported so far, the occurrence of this pathogen in seafood and the coastal-marine environment is a definite health concern. In order to understand the potential pathogenicity of *A. butzleri*, it is important to study the composition of virulence genes linked to the pathogenicity. The occurrence of the ten putative VGs in this set of 147 *A. butzleri* isolated from various origins is shown in Table 1. Among *A. butzleri* isolated from fish, shellfish and coastal water samples, six virulence genes namely *mviN* (100%), *cj1349* (97.2%), *ciaB* (95.9%), *tlyA* (91.8%), *pldA* (91.1%) and *cadF* (89.7%) were very predominant, being present in 118 (80.8%) of 147 isolates (Table 1). When the sources of isolates were considered, 88.8% of fish isolates, 76.9% of the isolates from coastal water and 65% of the isolates from shellfish harbored 6 virulence genes.

Table 1
Distribution of putative virulence genes in *Arcobacter butzleri*.

Species	Origin	No. of isolates	No. (%) of isolates generating specific gene amplicon									
			cadF	*ciaB*	*cj1349*	*mviN*	*pldA*	*tlyA*	*hecA*	*hecB*	*irgA*	*iroE*
A. butzleri	Fish[a]	81	75 (92.5)	81 (100)	81 (100)	81 (100)	79 (97.5)	78 (96.2)	8 (9.8)	15 (18.5)	12 (14.8)	10 (12.3)
	Shellfish	40	33 (82.5)	37 (92.5)	37 (92.5)	40 (100)	31 (77.5)	32 (80)	2 (5)	9 (22.5)	6 (15)	5 (12.5)
	Water[b]	26	24 (92.3)	23 (88.4)	25 (96.1)	26 (100)	24 (92.3)	25 (96.1)	6 (23)	4 (15.3)	8 (30.7)	4 (15.3)
Total		147	132 (89.7)	141 (95.9)	143 (97.2)	147 (100)	134 (91.1)	135 (91.8)	16 (10.8)	28 (19)	26 (17.6)	19 (12.9)

[a] One isolate carried all ten putative virulence genes.
[b] Two isolates carried all ten putative virulence genes.