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ORIGINAL ARTICLE

Incidence of *Arcobacter* spp. in fresh seafood from retail markets in Mumbai, India

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Abstract The occurrence of *Campylobacter*-like bacteria in fresh fish sold in retail markets of Mumbai was studied. Microaerophilic bacteria isolated following enrichment in Bolton broth and selective plating on Preston blood agar were subjected to biochemical identification followed by polymerase chain reaction to detect *Campylobacter*, *Arcobacter* and *Helicobacter* genera. *Arcobacter* spp. was isolated from 10 (25 %) of the 40 seafood samples analysed. Based on the comparison of partial 16S rDNA gene sequence, the *Arcobacter* isolates of this study were identified as *Arcobacter butzleri*, *A. skirrowii* and *A. cibarius*, with *A. butzleri* being the predominant species. The presence of arcobacter represents a health risk and requires further study to determine the sources of contamination of seafood and the pathogenic potential of *Arcobacter* spp.

Keywords *Arcobacter* · Microaerophiles · Seafood · PCR

Introduction

Bacteria of the genera *Campylobacter*, *Arcobacter* and *Helicobacter* are microaerophilic, Gram-negative, non-spore forming, spiral-shaped bacteria that are motile by means of polar flagella and constitute a phylogenetically distinct group referred to either as rRNA superfamily VI or as the epsilon division of the class Proteobacteria (Vandamme et al. 1991). These genera share a high degree of commonality with respect to their cultural characteristics, genetic composition and pathogenicity (Marshall and Warren 1984; Vandamme et al. 2000). The arcobacters distinguish themselves from *Campylobacter* spp. by being able to grow in the presence of atmospheric oxygen (aerotolerant) and at 15–37 °C (Neill et al. 1985). First isolated from aborted bovine and later from porcine fetuses, the genus *Arcobacter* has 18 species of which *A. butzleri*, *A. cryaerophilus*, *A. cibarus* and *A. skirrowii* are considered as human pathogens and have been isolated from cases of gastroenteritis, endocarditis, peritonitis and bacteraemia as well as from apparently healthy humans (Ellis et al. 1977; Ho et al. 2006; Collado and Figueras 2011; Levican and Figueras 2013). Livestock animals and birds harbour *Arcobacter* spp. and their isolation has been reported from both healthy and debilitated animals with mastitis, septicaemia, reproductive abnormalities and abortion (Kiehlbauch et al. 1991; Anderson et al. 1993; Schroeder-Tucker et al. 1996; Patyal et al. 2011). Several studies have isolated *Arcobacter* spp. from varieties of foodstuffs of animal origin, shellfish and water (de Boer et al. 1996; González et al. 2000; Kabeya et al. 2003; Scullion et al. 2006; Van Driessche and Houf 2007). Cases of human infection via contaminated drinking water, person-to-person transmission and meat have been reported (Collado and Figueras 2011).

Apart from meats of animal origin, *Arcobacter* spp. has been isolated from fish, shellfish and the aquatic environment. A high prevalence of *Arcobacter* spp. in clams and mussels has been reported (Collado et al. 2009). In the marine environment, culturable and non-culturable forms of arcobacters are present either as free living or in association with plankton (Fera et al. 2004). Recently, several new species of *Arcobacter* such as *A. bivalviorum*, *A. venerupis*, *A. molluscorum* sp. nov. and *A. cloacae* have been described from shellfish (Levican et al. 2012, 2013).

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Very few studies have investigated the occurrence of *Arcobacter* in animals, food and water and their involvement in human infections in India (Kownhar et al. 2007; Jiang et al. 2010; Patyal et al. 2011). In a study by Patyal et al. (2011), pig faeces showed the highest prevalence of *Arcobacter* spp. followed by seafoods. The presence in seafood of arcobacters such as *A. butzleri* that can cause human infections is of concern. Therefore, in the present study, we sought to investigate the occurrence of *Campylobacter*-like species in fresh seafood, especially known human pathogens belonging to the genera *Campylobacter*, *Helicobacter* and *Arcobacter*. *Arcobacter* spp. alone were isolated and identified from a large background flora of microaerophilic bacteria.

Material and methods

Sample collection and preparation

A total of 40 fresh fish samples were collected from different sources, of which 14 were from fish landing centres, 16 from wholesale markets and 10 from supermarkets in Mumbai, India. The samples included both pelagic and demersal fish. The samples were placed in sterile plastic bags (Hi-Media, Mumbai, India) containing ice and transported to the laboratory in an insulated box for further processing within 1 h of collection. Muscle and gut of fish were dissected out separately on a surface-sterilised stainless steel tray. Individual portions from at least five specimens were pooled on a sterile Petri dish and 10 g of the pooled sample was processed for bacteriological analysis.

Isolation of microaerophilic bacteria

The enrichment was carried out in Bolton broth in two steps; first, the samples were subjected to pre-enrichment in Bolton broth supplemented with laked horse blood (Oxoid, Basingstoke, UK) without antibiotics and second, a selective enrichment in the same broth containing antibiotics (20 mg L^{-1} each of cefoperazone, vancomycin and trimethoprim, 50 mg L^{-1} of cycloheximide). Briefly, 10 g of the fish samples was mixed with 90 mL Bolton broth without antibiotics and incubated for 6–8 h at 37 °C. Following this, 10 mL pre-enriched sample was added to 90 mL Bolton broth with antibiotics and 5 % (v/v) defibrinated horse blood (Oxoid). The broth was incubated for 48 h at 37 °C under microaerophilic conditions. For isolation of *Campylobacter*-like bacteria, two loopfuls were streaked on Preston blood agar containing antibiotics (5000 IU L^{-1} polymyxin B, 10 mg L^{-1} each of rifampicin and trimethoprim, 100 mg L^{-1} cycloheximide) and incubated at 37 °C for 48 h. For isolation of microaerophilic bacteria belonging to the genus *Helicobacter*, the homogenised samples were serially diluted in physiological saline (0.85 % NaCl w/v) and 0.1 mL volumes were spread plated on Columbia blood agar plates in duplicates. All incubations were done under a microaerophilic atmosphere generated by Anaerocult C (Merck, Mumbai, India). The bacterial colonies picked from the Columbia blood agar and Preston blood agar plates were purified on Luria Bertani (LB) agar and subjected to a series of biochemical tests shown in Table 1.

Molecular identification of the isolates

DNA was extracted from the isolates using a DNeasy DNA extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For PCR detection of the *Campylobacter-Arcobacter-Helicobacter* (CAH) group of bacteria, primers CAH16S1a (AATACATGCAAGTCGAACGA) and CAH16S1b (TTAACCCAACATCTCACGAC), which amplify a 1004-bp portion common to the 16S rDNA genes of the CAH group were used (Marshall et al. 1999). Primers previously described for *Campylobacter jejuni* (Linton et al. 1997) and *Helicobacter pylori* (Dore et al. 2001) were used for their PCR detection. For the detection of *Arcobacter* spp., primers Arco-I (AGAGATTAGCCTGTATTGTAT) and Arco-II (TAGCATCCCCGCTTCGAATGA) were used to amplify a 1202-bp region of the 16S rDNA gene as previously described (Harmon and Wesley 1996). The amplified products were analysed by gel electrophoresis, stained with ethidium bromide and photographed (Gel doc, Bio-Rad, Hercules, CA). PCR products obtained with *Arcobacter*-specific primers were purified using a PCR purification kit (MP Biomedicals, Santa Ana, CA) and sequenced (Bioserve Biotechnologies, Secunderabad, India).

Results

A total of 240 isolates was obtained from all the samples analysed, of which 10 were Gram negative, motile and typically helical in shape under the microscope. These isolates were subjected to phenotypical characterisation using the biochemical tests listed in Table 1. However, all ten isolates could grow aerobically, suggesting that they might be neither *Campylobacter* nor *Helicobacter*. Nucleotide BLAST analysis of 16S rDNA gene sequences amplified by CAH group-specific primers identified all of them as *Arcobacter* spp., with a sequence similarity of 98–100 % with GenBank sequences (Table 1) (Fig. 1). Of ten isolates, five were *A. butzleri*, four were *A. cibarius* and one was *A. skirrowii*. These three different species were distinguishable biochemically based on nitrate reduction (Table 1). *A. butzleri* and *A. cibarius* reduce nitrate while *A. skirrowii* does not. In addition, as opposed to the other two *Arcobacter* species

Table 1 Characteristics of *Arcobacter* spp. isolated in this study

Isolate	Catalase	Oxidase	Urease	Nitrate reduction	Hippurate hydrolysis	H_2S on TSI	Indoxyl acetate hydrolysis	Growth in 1 % glycine	Growth in 4 % NaCl	Growth on MacConkey agar	CAH-PCR	Blast identity	Accession no.
A-12	+	+	−	+	−	−	+	−	−	+	+	*Arcobacter butzleri*	KF527839
A-14	+	+	−	+	−	−	+	−	−	+	+	*Arcobacter butzleri*	KF527840
A-74	+	+	−	+	−	−	+	−	−	+	+	*Arcobacter butzleri*	KF527841
A-142	+	+	−	−	−	−	+	−	−	+	+	*Arcobacter cibarius*	KF527844
A-167	+	+	−	−	−	−	+	−	−	+	+	*Arcobacter cibarius*	KF527847
A-172	+	+	−	−	−	−	+	−	−	+	+	*Arcobacter cibarius*	KF527845
A-223	+	+	−	−	−	−	+	−	−	+	+	*Arcobacter cibarius*	KF527846
A-289	+	+	−	+	−	−	+	−	−	+	+	*Arcobacter butzleri*	KF527842
A-322	+	+	−	+	−	−	+	−	−	+	+	*Arcobacter butzleri*	KF527843
A-374	+	+	−	+	−	−	+	−	+	−	+	*Arcobacter skirrowii*	KF527848

TSI Triple sugar iron, *CAH-PCR* PCR detection of *Campylobacter-Arcobacter-Helicobacter* group of bacteria

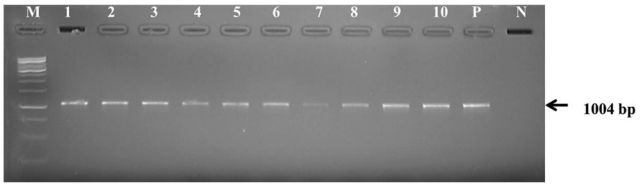

Fig. 1 Agarose gel electrophoresis of *Campylobacter-Arcobacter-Helicobacter* (CAH) group amplification products. Lanes: *M* GeneRuler 1 kb DNA ladder (Fermentas); *1–5 Arcobacter butzleri*; *6–9 Arcobacter cibarius*; *10 Arcobacter skirrowii*; *P* positive control *Helicobacter pylori*; *N* negative control

(Table 1), *Arcobacter skirrowii* grows at 4 % NaCl but does not grow on MacConkey agar.

Table 2 shows the seafood sample types that harbored *Arcobacter* spp. The ten isolates of *Arcobacter* obtained in this study were from equal numbers of samples from a total of 40 samples analysed. Of these positive samples, six were from the fish landing centre, and two each from wholesale and retail markets. Different portions of fish, namely, gut, skin and muscle, yielded *Arcobacter* spp., and no specific association of the bacterium with specific isolation site could be established (Table 3). Of five isolates of *A. butzleri*, three were isolated from the gut and two were from the muscle of marine fish. Two each of the four isolates of *A. cibarius* were from the muscle and gut, respectively, while the only isolate of *A. skirrowii* was obtained from the gut of fish.

Discussion

Initially classified as "aerotolerant campylobacters", arcobacters share a high degree of genetic and phenotypic similarity with campylobacters and are also implicated in human illnesses such as gastroenteritis and septicaemia (Taylor et al. 1991; Vandamme et al. 1991; Lerner et al. 1994; Yan et al. 2000; On 2001). Contamination of the aquatic environment with animal and poultry wastes has raised concerns regarding fish and shellfish being vehicles for *Arcobacter* dissemination, stressing the need for risk assessment (Lee et al. 2012). Shellfish such as clams and oysters are natural reservoirs of *Arcobacter* spp. and therefore could be potential sources of human infections (Collado and Figueras 2011). Shellfish have also been the source of several new species of *Arcobacter* of unknown pathogenic potential (Levican et al. 2012, 2013). In our study, biochemical and molecular assays helped to precisely identify different species of *Arcobacter* (Table 1). We analysed gut and muscle portions of fish separately for the presence of *Arcobacter* spp. While both *A. butzleri* and *A. cibarius* were isolated from the muscle and gut of the samples, the only isolate of *A. skirrowii* was obtained from the gut of a fish (Table 3). Several past studies have reported the occurrence of *Arcobacter* spp. in fresh and coastal waters, sewage and activated sludge (Fera et al. 2004;

Table 2 Distribution of *Arcobacter* spp. in fresh fish from different sources

Sample source	No. of samples positive for *Arcobacter* spp.			
	A.butzleri	*A.skirrowii*	*A.cibarius*	Total number (%) of positive samples
Landing centre (14)	2	0	4	6 (42.8)
Wholesale market (16)	1	1	0	2 (12.5)
Supermarket (10)	2	0	0	2 (20)
Total (40)	5	1	4	10 (25)

Morita et al. 2004; Pejchalová et al. 2008; Rice et al. 1999; Levican et al. 2013; Merga et al. 2014). Although we did not determine the prevalence of *Arcobacter* spp. in coastal waters, it may be predicted that the contamination of marine fish could have occurred from coastal water during fish landing or when near-shore water was used to wash the fresh fish catch. Other notable sources of contamination of seafood with *Arcobacter* spp. are the fresh water used to wash fish, and ice prepared from non-potable water.

Very few reports are available on the occurrence of *Arcobacter* spp. in seafood. A recent study has reported the presence of 11 different species of *Arcobacter* in mussels, clams and oysters, with *A. butzleri* being the most dominant species (Levican et al. 2014). This study found that the incidence of *Arcobacter* spp. was higher during periods of warmer water temperature. In Northern Spain, another study found *Arcobacter* spp. in 73.3 % of shellfish samples analysed (Nieva-Echevarria et al. 2013). Patyal et al. (2011) isolated *Arcobacter* spp. from 17.33 % of seafood samples in India, but detected *Arcobacter* DNA in 21.33 % of seafood samples by direct PCR on enrichment broths.

The efficacy of different enrichment broths and selective agar combinations for the efficient isolation of *Arcobacter* spp. from different food types has been tested (Johnson and Murano 1999). Selective agents used for *Campylobacter*-like bacteria do not restrict the growth of *Arcobacter*. The *Arcobacter* selective medium (de Boer et al. 1996) uses a selective supplement containing cefoperazone, trimethoprim, piperacillin and cycloheximide, whereas the Bolton broth used for pre-enrichment in the present study contained the selective agents cefoperazone, trimethoprim, vancomycin and cycloheximide. Several studies indicate resistance of *Campylobacter* to Piperacillin (Griggs et al. 2009), indicating that such selective media may not restrict *Campylobacter*. However, our study did not target *Arcobacter* specifically but was aimed at isolating *Campylobacter*-like bacteria, and the results suggest that the composition of the medium as well as the antibiotic supplements used, support the growth of *Arcobacter* spp. as well. The Anaerocult C used for creating specific microaerophilic conditions also allows the growth of aerotolerant arcobacters. However, a recent study has reported that the recovery of *Arcobacter* spp. is higher under aerobic conditions than under microaerophilic conditions (Levican et al. 2014). Based on this, it is possible that the actual incidence of *Arcobacter* in seafood in India may be much higher than the levels found in this study using only microaerophilic conditions.

Members of the genus *Arcobacter* have attracted attention in recent years as emerging water- and food-borne pathogens. *A. butzleri*, *A. cibarius*, *A. cryaerophilus* and

Table 3 Description of the samples, sources and sites of isolation of different *Arcobacter* spp.

Fish species	Portion	Sample source	*Arcobacter* spp. isolated
Cynoglossus cynoglossus	Muscle	Landing centre	*A. butzleri*
Trichiurus lepturus	Muscle	Landing centre	*A. butzleri*
Sardinella longiceps	Gut	Wholesale market	*A. butzleri*
Sardinella longiceps	Gut	Supermarket	*A. butzleri*
Rastrelliger kanagurta	Gut	Supermarket	*A. butzleri*
Coilia dussumieri	Gut	Landing centre	*A. cibarius*
Coilia dussumieri	Muscle	Landing centre	*A. cibarius*
Pampus argenteus	Gut	Landing centre	*A. cibarius*
Pampus argenteus	Muscle	Landing centre	*A. cibarius*
Protonibea diacanthus	Gut	Wholesale market	*A. skirrowii*

A. skirrowii have been associated with gastrointestinal diseases and bacteraemia in humans. *A. butzleri* is considered a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF 2002). With improvements in isolation techniques, a better understanding of *Arcobacter* distribution in seafood and the environment will emerge. Further studies are needed to elucidate the virulence factors, antibiotic resistance and survival of *Arcobacter* spp. in low-temperature-preserved seafood.

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