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Detection, identification, and antimicrobial susceptibility of *Arcobacter* spp. isolated from shellfish in Spain

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Abstract

This work aimed to determine the presence of *Arcobacter* spp. in shellfish and to determine its susceptibility to quinolones. One hundred samples (41 mussels, 37 clams, and 22 cockles) were purchased from different local retail shops in Valencia, Spain from September 2013 to June 2015. All samples were analyzed simultaneously by culture, after an enrichment step, and by PCR, directly and after enrichment. The susceptibility to levofloxacin and ciprofloxacin of the isolates was tested using the disk-diffusion test and E-test strips method. To clarify the mechanism of quinolone resistance, a fragment of the quinolone resistance-determining region (QRDR) of the *gyrA* gene was sequenced. 37 samples were positive and 49 isolates were obtained by culture, and *Arcobacter* spp. DNA was detected in 32% of the samples by PCR. However, after 48 h enrichment, the number of positive samples increased, and 68 of the 100 samples yielded the specific *Arcobacter* spp. PCR product. In addition, 49 isolates were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The most commonly found species was *A. butzleri* (25 isolates, 51.03%) followed by *A. cryaerophilus* (19 isolates, 38.77%) and *A. defluvii* (5 isolates, 10.20%). Only three isolates of *A. butzleri* were resistant to both antibiotics. A mutation C to T transition in the position 254 of the *gyrA* gene was present in the three resistant isolates. This study confirms that pathogenic arcobacters are frequently found in edible shellfish samples. Moreover, this is the first time that *A. butzleri* and *A. cryaerophilus* have been isolated from cockles.

Introduction

The *Arcobacter* genus belongs to the epsilon subdivision of proteobacteria and comprises Gram-negative, curved and usually motile bacilli, which can grow microaerobically and aerobically. They differ from the *Campylobacter* species in the ability to grow under aerobic conditions and temperatures below 30°C (Ünver *et al*., 2013).

This genus includes 23 species that have been recovered from different hosts and environments, such as sea water, waste water, plants, animal and human feces, or animal foodstuffs (Hsu & Lee, 2015). Some *Arcobacter* species are considered emergent-water and food-borne pathogens (Collado & Figueras, 2011). *A. butzleri*, *A. cryaerophilus,* and

A. skirrowii, have been associated with enteritis, colitis, and septicaemia (Van den Abeele *et al*., 2014). *A. butzleri* is the most commonly isolated species and has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (Collado *et al*., 2009).

In industrialised countries, foods of animal origin are considered the main transmission source of this bacterium to humans, especially if food are consumed raw or are poorly cooked (Serraino *et al*., 2013). Although some *Arcobacter* species have been primary isolated from shellfish (Figueras *et al*., 2011a and b; Levican *et al*., 2015), few studies have determined the presence of pathogenic *Arcobacter* spp. *A. butzleri* and *A. cryaerophilus* have been detected in bivalve mollusks such as clams, baby clams, oysters, and mussels (Collado *et al*., 2009; Fernandez *et al*., 2015). Despite the fact that no outbreaks have been related to the consumption of shellfish, there could be a plausible transmission route of virulent *Arcobacter* spp. strains to humans, as they are usually consumed in the raw state or under cooked (Collado *et al*., 2014). More studies are needed in order to establish the prevalence of pathogenic arcobacters in shellfish and the actual risk its presence poses to food safety.

Isolation of *Arcobacter* spp. from environmental and food samples is difficult and available techniques usually yield false negative results, thereby underestimating the actual presence of these bacteria in samples (Rahimi *et al.*, 2012). PCR is more sensitive than culture and provides more accurate information about contamination levels (González *et al.*, 2007); however, culture is necessary for isolation, identification, and characterization of *Arcobacter* spp. present in samples.

Phenotypic identification of *Arcobacter* species is problematic due to their low metabolic activity and the atypical reactions of some isolates (Atabay *et al*., 2006). Several DNA-based techniques have been described for identifying some *Arcobacter* species (Levican & Figueras, 2013) but none are able to differentiate all the species. The 16S rRNA-RFLP method developed by Figueras *et al*. (2012), which has been used in this work, is considered the most suitable method (Levican & Figueras, 2013).

Quinolones are first-line drugs for the treatment of infection by campylobacteria but very little is known about the resistance levels to these antibiotics among the *Arcobacter* species (Ünver *et al*., 2013). Moreover, resistance to quinolones could be due to a mutation in the QRDR region of the *gyrA* gene (Abdelbaqi *et al*., 2007) but this hypothesis has not yet been confirmed, due to the low number of resistant strains in which this mutation has been studied.

The aim of this study was to detect and identify pathogenic *Arcobacter* spp. from shellfish using culture and molecular methods, as well as to assess antimicrobial susceptibility of *Arcobacter* isolates to ciprofloxacin and levofloxacin and their possible resistance mechanisms.

Materials and methods

Sample preparation and enrichment

We processed 100 shellfish samples purchased in 11 different local retail shops from the city of Valencia (Spain) from September 2013 to June 2015. Samples were taken periodically, with approximately 4 samples per month. The study included 41 mussels (*Mytilus chilensis*), 37 clams (*Protothaca thaca*), and 22 cockles (*Cerastoderma edule*). Taxonomic identification of samples was recorded from information provided in commercial labels. Samples were transported to the laboratory stored at 4°C and analyzed within 3 h of purchased.

For detection by culture, 10 g of each shellfish sample was mixed with 90 mL (1:10 wt/vol) of *Arcobacter* broth (Oxoid CM0965, UK) for 2 min in a homogenizer (Stomacher Lab-Blender 400, Seward Medical, UK). Twenty mL of each homogenized sample were inoculated into 20 mL of *Arcobacter* broth with double concentration of Cefoperazone-Amphotericin B -Teicoplanin (2CAT) selective supplement (Oxoid SR0174E, UK). The samples were incubated at 37±1°C under microaerobic conditions using CampyGen sachets (Oxoid CN0035, UK) for 48±2 h (Atabay & Corry, 1997).

Arcobacter spp. detection and isolation

After enrichment, a 100 µL aliquot of the broth was placed onto a 0.45 µm cellulose membrane filter, which was deposited on the surface of *Arcobacter* agar plates with 5% defibrinated sheep blood, according to the technique described by Atabay & Corry (1997). These filters were removed after one hour of incubation at 30°C under aerobic condition, and the plates were incubated under microaerobic conditions at 37±1°C for 48±2 h. Each assay was performed in duplicate. From each plate, one to four presumptive *Arcobacter* spp. colonies (grey-white and round small clear) were selected and checked by Gram stain.

For PCR detection, aliquots of 150 µL from homogenized samples were analyzed before and after the 48 h enrichment period. The aliquots were centrifuged for 2 min at 12000–16000 xg to pellet the bacteria and DNA was subsequently extracted using a DNA extraction Kit (GeneElute Bacterial Genomic DNA, Sigma-Aldrich, Missouri, USA), following the manufacturer's instructions.

A specific 23S rRNA PCR (Bastyns *et al.*, 1995) was performed on all the samples, by using primers and thermal cycling protocols specific for *Arcobacter* genus (Table 1). DNA from *Arcobacter butzleri* DSM 8739 was used as a positive control. PCR products were visualized on a 1.2% agarose gel stained with Red Safe (Ecogen 21141, Barcelona, Spain) in a UV transilluminator. Each assay was performed in duplicate.

Speciation of Arcobacter spp. isolates

To identify presumptive isolates at the genus level, pure cultures of each isolate were grown and re-suspended in 300 µL of Tris–EDTA (TE) buffer. After that, DNA extraction was developed and processed by PCR as described below.

The species identification was carried out using the 16S rRNA-RFLP method described by Figueras *et al*. (2012) (Table 1). The amplicon was observed by electrophoresis on a 1.2% agarose gel in a transilluminator. The PCR product was purified using the GenEluete PCR Clean-Up Kit (Sigma-Aldrich NA1020, Missouri, USA) according to the manufacturer's instructions. After purification, 16S rRNA amplicons were digested with 20 U of the endonuclease *Mse*I and 2 µL of 10X buffer R, incubating the mixture at 65°C for 5 h. This enzyme is able to detect 10 out of the 17 species described until 2012. Additionally, to discriminate between *A. defluvii* and *A.* suis, we used 20 U the endonuclease *Bfa*I with 2 µL of 10 X buffer and similar incubation conditions as the first digestion. To distinguish *A. butzleri* from *A. thereius and A. trophiarum*, we performed a digestion with endonuclease *Mnl*I (Thermo Fisher Scientific, Germany): 20 U of the enzyme was mixed with 2 µL of 10 X buffer G and incubated at 37°C for 5 hours. In each digestion 10 µL of purified PCR products were used in a total volume of 32 µL.

Restriction fragments were separated by electrophoresis on 3.5% agarose gels (Roche, 03573788) prepared in 1 X TAE buffer at 90 volts for 2.5 hours. A 50-bp ladder (Thermo Fisher Scientific, SM0373, Germany) was used as a molecular weight marker. The gels were stained with Gel Red Nucleotide Acid (Biotium, 10000X, USA) according to the manufacturers' instructions. Finally, gels were photographed on a UV transilluminator.

For the validation of species-specific PCR-RFLP patterns obtained from each isolate, six reference strains were used (Table 2).

Computer analysis

In order to obtain the restriction patterns of each strain, a computational simulation (*in silico* assay) was developed by sequencing the 16S rRNA gene of the species *A. butzleri, A. cryaerophilus 1A and 1B, A. defluvii, A. mytili, A. molluscorum, A. ellisii, A. suis*, *and A. thereius* type strains (http://www.ncbi.nlm.nih.gov/nuccore/FJ573216.1). The 1026-bp fragment from each type strain was analyzed by REBASE (http://rebase.neb.com/rebase/rebtools.html), using the restriction enzymes *Mse*I, *Bfa*I, and *Mnl*I. The results were compared with experimental assays.

Antimicrobial susceptibility

Antimicrobial susceptibility analysis of the *Arcobacter* spp. isolates was developed using the disc-diffusion (BD, USA) and E-test strips (BioMérieux, France) methods. The antimicrobial agents used were ciprofloxacin and levofloxacin discs (5 µg/disc) and E-test (0.002 to 32 µg/mL).

The identified isolates were sub-cultured on *Arcobacter* agar under microaerobic conditions at 37°C for 48 h. Several colonies of fresh pure culture from each isolate were suspended in *Arcobacter* broth until the turbidity was adjusted to match the McFarland 0.5 standards. Bacteria from each suspension were inoculated onto *Arcobacter* agar using a sterile cotton-tipped swab and the plates were left stand for a few minutes. Thereafter, the discs and E-test strips were applied to the agar surfaces. The plates were incubated in

microaerobic atmosphere at 37±1°C for 48±2 h. After incubation, the diameter of the inhibition zones surrounding the discs were measured in millimeters, and the point where the elliptical zone of inhibition intersected the Minimum Inhibitory Concentration (MIC) scale on the strip was read. The susceptibility patterns (resistance/sensitivity) of the isolates were determined according to criteria for *Campylobacter* of the National Committee for Clinical Laboratory Standards (CLSI 2010, M45-A2) since there is not a standard for *Arcobacter* spp. and this genus belongs to the same family as *Campylobacter*. According to these criteria, a disc diffusion zone ≤6 mm and a MIC value ≥4 µg/mL indicates resistance, while a MIC value ≤1 and a disc diffusion zone of >6 mm indicates susceptibility. *A. butzleri* DSM 8739 type strain (susceptible to quinolones) was used as a control. All assays were performed in duplicate.

Analysis of the mechanism of quinolone resistance

The PCR assay described by Abdelbaqi *et al*. (2007) was performed to determine the mechanism of quinolone resistance of the resistant isolates (Table 1). The assay was developed both for the resistant isolates and 3 susceptible isolates as well as the *A. butzleri* DSM 8739 type strain as a control to compare the results. The PCR product was purified by using a genomic DNA extraction Kit (GeneElute Bacterial Genomic DNA Kit, Sigma-Aldrich, USA) following the manufacturer's instructions. Purified PCR product was sequenced by Instituto de Biología Molecular y Celular de Plantas, Universitat Politècnica de València (IBMCP-UPV, Valencia, Spain).

Results and discussion

Arcobacter spp. detection and identification using PCR

Arcobacter spp. were detected in 68 out of the 100 samples (68%) using conventional PCR after 48 h enrichment in AB supplemented with 2CAT at 37°C under microaerophilic conditions. The highest number of contaminated samples was found in clams (75.67%, 28/37), followed by mussels (73.17%, 30/41) and cockles (45.45%, 10/22) (Table 3). These levels are high compared to those reported by other studies, which range from 16.7% (Levican *et al*., 2014) to 40.5% (Collado *et al*., 2014). Our results suggest that shellfish may be an important reservoir for arcobacters and that this type of food could be a possible source of *Arcobacter* spp. to humans.

It has previously been reported that the combination of PCR with a prior enrichment treatment of samples increases the level of viable cells, providing better detection results (Collado *et al*., 2009), and our results confirm these data. The detection levels without previous enrichment were lowest and only 32 samples (32%) were positive (Table 3), suggesting high contamination in these samples.

Three samples were positive by direct PCR; however, *Arcobacter* spp. were not detected after 48 h enrichment, likely due to the fact that DNA in the initial broth came from dead cells and was diluted after enrichment (Denis *et al*., 2001). Additionally, isolate M99 was recovered by culture from a sample that was PCR negative, both before and after

enrichment. These results could be due to the presence of inhibitor substances in the sample or in the enrichment broth (Klancnik *et al*., 2012).

Arcobacter spp. isolation and identification using culture

Arcobacter spp. were isolated from thirty-seven out of 100 shellfish samples (37%). The highest detection levels were found in mussels (41.46%), followed by clams (40.54%) and cockles (22.73%). In total, forty-nine isolates were obtained, 21 from mussels, 20 from clams, and 8 from cockles (Table 3). The contamination levels for mussels and clams are similar to those reported by other studies in Spain (Collado *et al*., 2014; Levican *et al*., 2014). To our knowledge, no comparable data on *Arcobacter* spp. in cockles are available.

As expected, the number of PCR positive samples after 48 h enrichment was higher than those obtained by culture. PCR has largely proved to provide much more positive results than culture when environmental or food samples are examined (Gonzalez *et al.*, 2007). In many cases, these results are due to the low number of viable cells or to the presence of Viable But not Culturable (VNC) cells in the sample, which cannot be recovered by culture (Ramamurthy *et al.*, 2014).

Identification of Arcobacter spp. isolates using PCR-RFLP

All experimental 16S rRNA-RFLP patterns were similar to those obtained by computational *in silico* assay. The method used in this work allows the identification of up to 17 of the 23 species currently recognized. Three species were identified using the 16S rRNA-RFLP assay with *Mse*I, *Mnl*I, and *Bfa*I (Table 3); *A. butzleri* was the most prevalent (51.03%, 25/49). Similar levels have been reported by other studies as follows: 62% (Collado *et al*., 2014), 42% (Collado *et al*., 2009b), and 60.2% (Levican *et al*., 2014). *A. cryaerophilus* was the second species most frequently identified (38.77%, 19/49). Collado *et al*. (2014) identified this species in 21% of shellfish samples, while Levican *et al*. (2014) reported the lowest results (5.1%). In this study, 5 out of 49 isolates were identified as *A. defluvii* (10.20%); four of them were isolated from clams (Fig. 1). To our knowledge, this is the first report of *A. defluvii* isolation from clams. This species was firstly described from sewage (Collado *et al.*, 2011) and there is no information about its pathogenicity.

In this study, the two main *Arcobacter* pathogenic species, *A. butzleri* and *A. cryaerophilus*, were isolated. A large percentage of commercial samples are contaminated, raising the question of food safety. This is also the first time that *Arcobacter butzleri* and *Arcobacter cryaerophilus* have been isolated from cockles (Fig. 1), suggesting that this type of sample could be an important reservoir for this species.

Antimicrobial susceptibility of Arcobacter spp. isolates

Twenty-two out of the 25 *A. butzleri* isolates were susceptible to quinolones, while three (12 %) showed resistance to both levofloxacin and ciprofloxacin by disc diffusion tests and E-test strips. All isolates of *A. cryaerophilus* and *A. defluvii* showed susceptibility to both antibiotics.

These antimicrobials were selected because they are the first-line drugs for the treatment of *Campylobacter* infection (Vandenberg *et al*., 2006). Fera *et al*. (2003) and Akıncıoglu (2011) reported that fluoroquinolones are effective against *A. butzleri* and *A. cryaerophilus* environmental isolates, and similar results were reported by Vandenberg *et al*. (2006) and Mandisodza *et al*. (2012) when they analyzed strains isolated from clinical and human fecal samples. Our results are in accordance with previous reports, with 93.88% of the isolates being susceptible to both fluoroquinolones.

Analysis of the QRDR of the gyrA gene

From the three resistant *A. butzleri*, three susceptible isolates and the reference strain DSM8739, a 344-bp QRDR fragment of *gyrA* gene was amplified by PCR (Fig. 2). The sequencing of the PCR product revealed that the three resistant isolates carried a mutation in position 254 of the *gyrA* gene (C to T transition), while the susceptible isolates did not show the mutation. Mutations in the *gyrA* gene encoding the subunit of DNA gyrase are most often involved in quinolone resistance among Gram-negative bacteria (Aldred *et al*., 2014).

In conclusion, the results of this study indicate the importance of shellfish as a source of pathogenic *Arcobacter* spp., a question of food safety concern. To our knowledge, this is the first report of *A. butzleri* and *A. cryaerophilus* isolation from cockles and *A. defluvii* from clams, which shows the great variability of niches where this microorganism may be present. In addition, the presence of *A. butzleri* quinolone resistance in mollusks could pose a potential public health risk since fluoroquinolones are the most used antibiotics to treat this kind of infections.

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Table 1. Primers and PCR conditions

Gene /Primer Sequences	**PCR MIX**	**PCR Reaction**	**Product Length**	**Reference**
23S rRNA (*Arcobacter* genus): ARCO1: GTCGTGCCAAGAAAAGCCA ARCO2: TTCGCTTGCGCTGACAT	1 X NH_4 buffer 2 mM $MgCl_2$ dNTP´s 0.1 mM each 5 U *Taq* polymerase	Initial denaturation: 94°C for 5 min 27 cycles at: 94°C for 60 s 60ºC for 60 s 72ºC for 60 s Final extension: 72°C for 5 min	331-bp	(Bastyns *et al.*, 1995)
16S rRNA (PCR-RFLP): CAH1am: ACACATGCAAGTCGAACGA CAH1b: TTAACCCAACATCTCACGAC	1 X NH_4 buffer 1.5 mM $MgCl_2$ dNTP´s 0.2 mM each 5 U *Taq* polymerase	Initial denaturation: 94°C for 2 min 30 cycles at: 94°C for 30 s 52°C for 30 s 72°C for 90 s Final extension 72°C for 10 min	1026-bp	(Figueras *et al.*, 2012)
gyrA(QRDR Region): F–QRDR: TGGATTAAAGCCAGTTCATAGAAG R2–QRDR: TCATMGWATCATCATAATTTGGWAC	1X NH4 buffer 2 mM $MgCl_2$ dNTP´s, 0.2 mM each 2.5 U of *Taq* polymerase	Initial denaturation: 95°C for 5 min 35 cycles at: 95°C for 30 s 60°C for 30 s 72°C for 2 min Final extension 72°C for 5 min	344-bp	(Abdelbaqi *et al.*, 2007)

Table 2. List of reference strains of *Arcobacter* species

	Species	Source	Reference strains ID
1	*Arcobacter butzleri*	Human diarrheal stool	DSM 8739
2	*Arcobacter cryaerophilus 1A*	Brain of aborted bovine fetus	CECT 8222
3	*Arcobacter defluvii*	Sewage	CECT 7697
4	*Arcobacter mytili*	Mussels	CECT 7386
5	*Arcobacter molluscorum*	Mussels	CECT 7696
6	*Arcobacter ellisii*	Mussels	CECT 7837

Table 3. *Arcobacter* spp. detection in shellfish using PCR and culture

Sample	N° of samples	N° (%) of positive samples by PCR		N° (%) of positive samples by culture							
						A. butzleri		*A. cryaerophilus*		*A. defluvii*	
		By PCR before enrichment	By PCR after enrichment	N° positive samples (%)	N° isolates	N° samples	N° isolates (%)	N° samples	N° isolates (%)	N° samples	N° isolates (%)
Mussels	41	19 (46.34)	30 (73.17)	17 (41.46)	21	5	7 (33.34)	11	13 (61.90)	1	1(4.76)
Clams	37	9 (24.32)	28 (75.67)	15 (40.54)	20	7	11(55)	5	5 (25)	3	4(20)
Cockles	22	4 (18.18)	10 (45.45)	5 (22.73)	8	4	7(87.5)	1	1 (12.5)	0	0
Total	100	32	68	37	49	16	25 (51.03)	17	19 (38.77)	4	5 (10.20)

Legend to the figures

FIG. 1. 16S rRNA-RFLP patterns obtained for *Arcobacter* isolates using the endonuclease *Mse*I. Lanes M: 50-bp ladder; lanes 1 and 2: cockle isolates; lane 3: *A. cryaerophilus* CECT 8222; lanes 4 and 5: clam isolates; lane 6: *A. defluvii* CECT7697; lane 7 and 8: cockle isolates; lane 9: *A. butzleri* DSM8739.

FIG. 2. Amplified fragment of *gyrA* gene of shellfish isolates. Lanes M: 100-bp ladder; lanes 1, 2 and 3: resistant *A. butzleri* isolates; lanes 4, 5 and 6: susceptible *A. butzleri* isolates; lane 7: *A. butzleri* DSM8739; lane 8: negative control.

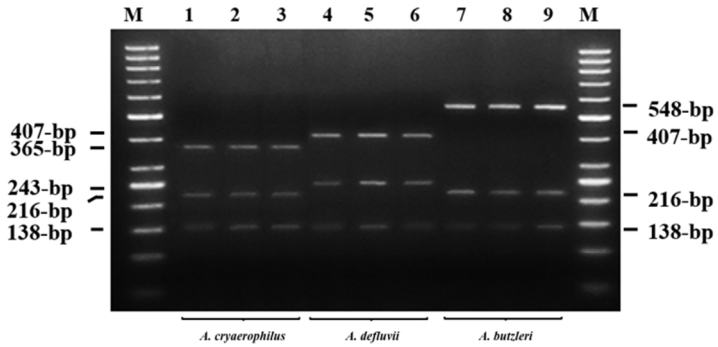

Figure 1

338x190mm (72 x 72 DPI)

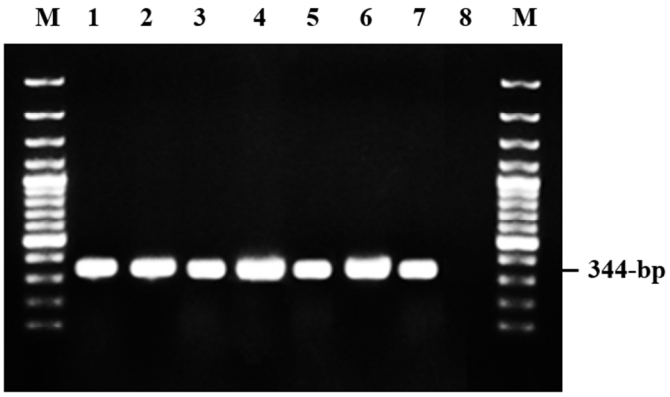

Figure 2

361x270mm (72 x 72 DPI)