Detection of *Arcobacter* spp. in *Mytilus galloprovincialis* samples collected from Apulia region

Elisabetta Bonerba,[1] Anna Mottola,[1] Antonio Parisi,[2] Angela Di Pinto,[1] Andrea Serraino,[3] Giancarlo Bozzo,[1] Federica Giacometti,[3] Edmondo Ceci,[1] Giuseppina Tantillo[1]

[1]Dipartimento di Medicina Veterinaria, Università di Bari, Valenzano (BA); [2]Istituto Zooprofilattico Sperimentale della Puglia e Basilicata, Putignano (BA); [3]Dipartimento di Scienze Mediche Veterinarie, Alma Mater Studiorum - Università di Bologna, Ozzano Emilia (BO), Italy

Abstract

The aim of the study was to evaluate the occurrence of *Arcobacter* spp. in 20 samples of *Mytilus galloprovincialis* purchased at fish markets in Apulia region. The detection of *Arcobacter* spp. was performed, after selective enrichment, on modified charcoal cefoperazone deoxycholate (mCCD) agar supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT). In 6 out of the 20 tested samples the presence of *Arcobacter* spp. was found and confirmed by genus-based polymerase chain reaction. All the isolates were identified as belonging to the species *Arcobacter butzleri* using 16S rDNA sequencing and BLAST online. The results represent the first report in Italy of *A. butzleri* detection in marketed *Mytilus galloprovincialis*. The survey underlines the epidemiological importance of *A. butzleri* as an emerging pathogen, and highlights that mussels should be considered as a potential cause of foodborne disease outbreak.

Introduction

Arcobacter spp. was proposed as a new genus in 1991 by Vandamme and De Ley who defined it as *aerotolerant campylobacter*. This genus belongs to the class *Epsilonproteobacteria* and to family *Campylobacteraceae* (Phillips, 2001; Levican *et al.,* 2014). *Arcobacter* are rod, gram negative, microaerophilic, non-spore forming, motile, curved and occasionally straight organisms which can grow between 15 and 39°C (González and Ferrús, 2011; Tabatabaei *et al.*, 2014). Currently, the genus includes 18 characterized species (Levican *et al*., 2014; Nieva-Echevarria *et al*., 2013), among them, *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* are considered as potential emerging food borne enteropathogens (Levican and Figueras, 2013) and have been associated with human and animal disease (Tabatabei *et al.,* 2014; Levican *et al*., 2014; Suelam, 2012). *A. butzleri* has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMFS, 2002) and as a significant zoonotic pathogen (Cardoen *et al.,* 2009). Moreover, *A. butzleri* has been recognised as a cause of traveller's diarrhea (Jiang *et al.,* 2010).

Potential routes of *Arcobacter* spp. infection in human may be associated to the consumption and/or manipulation of contaminated raw or poorly cooked food of animal origin (Collado and Figueras, 2011; Gonzales and Ferrús, 2011; Hausdorf *et al.,* 2011; Nieva-Echevarria *et al*., 2013). Furthermore, these bacteria are members of seawater microbiota, wastewater and drinking water reservoirs (Collado *et al.,* 2008). Studies carried out by Fera *et al.* (2004) suggest that *A. butzleri* arrives in seawater through polluted freshwater and that this organism survives in the marine environment by adhering to zooplankton.

Bivalve mollusks, due to their ability to concentrate microorganisms from contaminated water during their filter-feeding activities, are considered as an important health risk, because they are often eaten poorly cooked and/or raw (Collado *et al.,* 2009; Levican *et al.,* 2014; Ottaviani *et al*., 2013). Despite this important risk, worldwide only a few surveys investigated the presence of *Arcobacter* spp. in these products. In Italy, the occurrence of *Arcobacter* spp. in marketed shellfish has not been investigated yet; only Maugeri and colleagues (2000) detected *A. butzleri* and *A. nitrofigilis* in water and mussels collected from two brackish lakes near Messina, but the isolates were characterized only phenotypically.

The purpose of this study was to evaluate the presence of *Arcobacter* spp. in *Mytilus galloprovincialis* sampled at retail in Apulia region (Italy).

Materials and Methods

Sampling and sample processing

A total of 20 *Mytilus galloprovincialis* samples of average size (5±7 cm length) were collected between January and April 2014 from local fish markets of Apulia region, Italy. Each sample was individually packaged and kept in coolers. Mussels were aseptically prepared for analysis in accordance with the UNI EN ISO 6887-3 standard procedure (ISO, 2003). For each sample, 10 g of meat and intervalvar liquid were homogenized with 90 mL (1:10, wt/vol) of *Arcobacter* enrichment broth supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) (selective supplement SR0174E; Oxoid, Basingstoke, UK) in stomacher bags. The bags were closed and incubated at 30°C under aerobic conditions for 48 h, and then 200 µL of the broth was inoculated by passive filtration onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) supplemented with CAT selective supplement, following the procedure described by Collado *et al.* (2009). Subsequently, presumptive *Arcobacter* colonies (small colourless or beige to off-white, translucent, convex with an entire edge, Gram negative) were selected from each plate and transferred to blood agar at least three times to obtain pure cultures. Purified isolates were further subjected to biochemical analysis (catalase, oxidase and urease tests), microscopic examination, and genus-specific polymerase chain reaction (PCR).

DNA extraction and purification

DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Briefly, bacterial pellet was added to 50 µL ATL lysis buffer and 5.56 µL of Proteinase K (20 mg/mL) and incubated at 56°C for 2 h. After adding 55.6 µL AL buffer and 55.6 µL ethanol, the resulting mixture was applied to the DNeasy Mini spin column. The DNA, adsorbed onto the QIAamp silica-gel membrane during subsequent centrifugation steps at 6000 g for 1 min, was washed using 140 µL AW1 and 140 µL AW2 washing buffers. Finally, the DNA was eluted with 50 µL AE Elution Buffer (Qiagen). The type strains of *A. butzleri* (ATCC 49616) was used as

Correspondence: Anna Mottola, Dipartimento di Medicina Veterinaria, Università di Bari, Strada provinciale per Casamassima km. 3, 70010 Valenzano (BA), Italy.
Tel. +39.080.5443853 - Fax: +39.080.5443853.
E-mail: annamottola_2006@libero.it

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positive control. A negative extraction control (no added tissue) was included to verify the purity of the extraction reagents. The DNA concentration and purity were established by evaluating the ratio A260 nm/A280 nm using a Beckman DU-640B spectrophotometer.

Oligonucleotide primers

The oligonucleotide primers ARCOI (5'-AGA GAT TAG CCT GTA TTG TAT C-3') and ARCOII (5'-TAG CAT CCC CGT TTC GAA TGA-3') reported by Harmon and Wesley (1996) and synthesized by Primm Srl (Milan, Italy) were used.

Polymerase chain reaction assay

The PCR reactions were performed in a final volume of 25 µL, using 12.5 µL of HotStarTaq Master Mix 2X (Qiagen), containing 2.5 units of HotStarTaq DNA polymerase, 1.5 mM of $MgCl_2$ and 200 µL of each dNTP. Then, 1 µM of each oligonucleotide primer and 1 µL of DNA were added. The amplification profile involved an initial denaturation step at 95°C for 5 min, followed by 30 amplification cycles. Each amplification cycle consisted of 30 s at 94°C, 30 s at 51°C and 60 s at 72°C. A primer extension step (72°C for 10 min) followed the final amplification cycle. The positive and negative controls for the extraction were included. The PCR reactions were processed in a Mastercycler Personal (Eppendorf, Hamburg, Germany). All reactions were performed in duplicate.

Detection of amplified products

Polymerase chain reaction amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X TBE buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with Green gel Safe Nucleic Acid Stain, 10,000 X in water (Fisher Molecular Biology, Rome, Italy). A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf).

Polymerase chain reaction amplification and sequencing of 16S rDNA

The universal 16S rDNA primers, forward primer 8F (5 AGTTGATCCTGGCTCAG3′), and reverse primer 1492R (5 ACCTTGTTACGACTT3′) were used for PCR. The PCR amplification was carried out in a reaction mixture containing ~10 ng genomic DNA as template in 25 mL reaction volumes containing 10 pmol of each primer, 0.2 mM of each dNTP's, 1 U HotMaster Taq (Eppendorf), 2.5 mL of 10X HotMaster Taq Buffer (Eppendorf). The reaction conditions were an initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 52°C for 1 min and 72°C for 1 min. The PCR products were purified using Montage PCR filter units (Millipore, Billerica, MA, USA). Sequence reactions were carried out using BigDye 3.1 ready reaction mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequenced products were separated with a 3130 Genetic Analyzer (Applied Biosystems). Sequences were imported and assembled with the Bionumerics 7.1 software (Applied Maths, Sint-Martens-Latem, Belgium) and submitted to BLAST searches in GenBank (Altschul *et al.*, 1990)

Results

Microbiological analysis

Based on the phenotypic cultural characteristic, the morphological examination by the Gram staining, and the biochemical analysis performed on each sample analysed, a total of 10/20 presumptive *Arcobacter* species were isolated by mCCDA supplemented with CAT (Table 1). The colonies showing white to whitish-grey, small (2-4 mm) diameter, convex and opaque with entire edge, smooth, transparent/translucent were suspected as *Arcobacter* spp. colonies. Moreover, oxidase and catalase positive and urease negative test revealed presumptive *Arcobacter* spp. isolates.

Biomolecular analysis

The isolates were confirmed as Arcobacters by genus-based PCR. Polymerase chain reactions performed on each bacterial pellet samples gave positive results for *Arcobacter* species in 6/10 (Table 1). Sequence analysis of the amplified 16S rDNA revealed that all Arcobacters isolates have a complete (100%) homology with *A. butzleri*.

Discussion

This is the first report of *A. butzleri* detection in *Mytilus galloprovincialis* marketed in Apulia region. However, since only 20 samples were analysed, the reported results should be interpreted only as preliminary data and require further sampling and analytical investigations to determine the prevalence of *Arcobacter* spp. in Italian marketed mussels.

The importance of the genus *Arcobacter* is due to some species defined as emerging enterophatogens and potential zoonotic agents. The current state-of-the-art on the transmission of Arcobacters to human suggests that the potential routes are represented by food and water contaminated.

Among *Arcobacter* spp. isolated from food

Table 1. Prevalence of *Arcobacter butzleri* isolated by cultural method.

Samples	Positive samples on cultural media	Positive samples on genus-based PCR (n)	Species detected by sequencing
1	-	-	
2	-	-	
3	+	+	*A. butzleri*
4	+	+	*A. butzleri*
5	+	+	*A. butzleri*
6	-	-	
7	+	+	*A. butzleri*
8	+	-	
9	-	-	
10	+	-	
11	-	-	
12	-	-	
13	-	-	
14	+	+	*A. butzleri*
15	-	-	
16	+	-	
17	-	-	
18	+	+	*A. butzleri*
19	-	-	
20	+	-	
Total	10	6	

PCR, polymerase chain reaction.

and water, *A. butzleri* is the most prevalent species, followed by *A. cryoaerophilus*. *Arcobacter* spp. have been frequently isolated from products of animal origin (chicken, followed by pork, beef meat, raw milk and dairy products, lamb) (Ho *et al.,* 2006; Fernandez *et al.*, 2001; Maugeri et *al.*, 2000; Giacometti *et. al.*, 2013). Recently *Arcobacter* spp. were detected in fresh vegetables (Gonzales and Ferrús, 2011), but these foods are generally considered as safe and *Arcobacter* contamination levels seem to be rather lower than animal food products and waters. *Arcobacters* have been isolated from environmental waters, including surface waters, ground waters, rivers, lakes, sea water, sewage and from planktons (Fera *et al.*, 2004; Collado *et al.*, 2008; Ghane, 2014). In Italy, Fera *et al*. (2004) reported the detection of these microrganisms in seawater and plankton samples collected from the Strait of Messina. Collado *et al*. (2008) confirmed the association of *Arcobacter* with faecally polluted waters.

Comparing data on the rates of prevalence of the Arcobacters, isolated from different sources, is very difficult. This could be accounted for several reasons, including geographic and temporal variation of sampling. Most of all, the absence of standard diagnostic techniques should be considered as an obstacle, together with inconsistences in the sensitivity and specificity of the various protocols developed. Bivalve shellfish, as a result of their filter-feeding activity, can bioconcentrate pathogens, but few data about *Arcobacter* spp. occurrence in mussels have been published.

The prevalence of *A. butzleri* in shellfish and the reported incidence rates were 100% in clams and 41.1% in mussels (Collado *et al.,* 2009; Maugeri *et al*., 2000; Fernandez *et al.,* 2001; Romero *et al*., 2002). New species of the genus Arcobacter have been isolated and identified from shellfish: *A. mytili*, *A. molluscorum, A. ellisii*, *A. bivalviorum* and *A. venerupis* (Levican *et al*., 2013).

The results reported here demonstrate the presence of *A. butzleri* in mussels marketed in Apulia region, Italy. These preliminary data require additional investigation in order to assess the epidemiology of this emerging foodborne pathogen, determine the origin of bacterial contamination (*i.e*. marine production areas), and study the prevalence of Arcobacters in lamellibranch molluscs.

Conclusions

The occurrence of *A. butzleri* in Italian mussels, marketed in Apulia region, emphasises the need to investigate the impact of *Arcobacter* spp. on public health, including this source of exposure. Consuming shellfish might be an important health risk when considering that these products are traditionally eaten poorly cooked and/or raw. An effective national food control system is essential to protect the health and safety of consumers, but it must be accompanied by extensive research on emerging pathogens.

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