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

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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 mCCDA enrichment, on modified charcoal cefoperazone deoxycholate (mCCDA) agar supplement- ed 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 sequenc- ing 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 Ferris, 2011; Tabatabaei et al., 2014). Currently, the genus includes 18 char- acterized species (Levican et al., 2014; Nieva-Echevarría 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 (Tabatabaei et al., 2014; Levican et al., 2014; Suelam, 2012). A. butzleri has been classi- fied as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 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 consump- tion and/or manipulation of contaminated raw or poorly cooked food of animal origin (Collado and Figueras, 2011; Gonzales and Ferris, 2011; Hausdorf et al., 2011; Nieva-Echevarría et al., 2013). Furthermore, these bacteria are members of seawater microbota, 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 con- centrate 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 col- leagues (2000) detected A. butzleri and A. nitrofigilis in water and mussels collected from two brackish lakes near Messina, but the iso- lates were characterized only phenotypically.

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

Materials and Methods

Sampling and sample processing

A total of 20 Mytilus galloprovincialis sam- ples of average size (5±7 cm length) were col- lected 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 liq- uid were homogenized with 90 mL (1:10, wt/vol) of Arcobacter enrichment broth supple- mented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) (selective supplement SR0174E; Oxoix, Basingstoke, UK) in stom- acher bags. The bags were closed and incubat- ed 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 3 h. After adding 55.6 µL AL buffer and 5.56 µL ethanol, the resulting mixture was applied to the DNeasy Mini spin column. The DNA, adsorbed onto the QiAamp silica-gel membrane during subsequent centri- fugation 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

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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 GTG 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 MgCl₂, 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 30 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′-AGTTGATCCCTGTCGCTAG3′), and reverse primer 1492R (5′-ACCTGTTGATCAGTCTGG3′) were used for PCR. The PCR amplification was carried out in a reaction mixture containing ~10 ng genomic DNA as template in 25 µL 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).

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

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 enteropathogens 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...
and water, A. butzleri is the most prevalent species, followed by A. cryoaeophilus. 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 lakes, rivers, 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 inconsistencies in the sensitivity and specificity of the various protocols developed. Bivalve shellfish, as a result of their filter-feeding activity, can bioconcentrate Arcobacters, isolated from different sources, followed by A. butzleri, A. mytili, A. venerupis, A. bivalviorum, A. ellisi, 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. The occurrence of A. butzleri in Italian mussel species, 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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