International Journal of Food Microbiology 162 (2013) 82–88

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse

Susana Ferreira [a], Maria J. Fraqueza [b], João A. Queiroz [a], Fernanda C. Domingues [a,*], Mónica Oleastro [c]

[a] CICS-UBI–Health Sciences Research Centre, University of Beira Interior, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal
[b] Faculty of Veterinary Medicine, CIISA, TULisbon, Av. da Universidade Técnica, Pólo Universitário, Alto da Ajuda, 1300-477 Lisbon, Portugal
[c] National Institute of Health Dr. Ricardo Jorge, Department of Infectious Diseases, National Reference Laboratory for Gastrointestinal Infections, Av. Padre Cruz, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 14 August 2012
Received in revised form 29 November 2012
Accepted 4 January 2013
Available online 11 January 2013

Keywords:
Arcobacter butzleri
Poultry
Pulsed field gel electrophoresis
Antimicrobial resistance
Biofilm

ABSTRACT

The genus *Arcobacter* is an emerging pathogen associated with several clinical symptoms. This genus is widely distributed and has been isolated from environmental, animal, food and human samples, where poultry is considered the major source. In this study, forty three *Arcobacter butzleri* strains isolated from poultry and environment of a Portuguese slaughterhouse, were characterized by pulsed field gel electrophoresis (PFGE) and assessed for antimicrobial susceptibility and ability to form biofilms. PFGE patterns obtained using restriction enzymes *SmaI* and *SacII* revealed high genetic diversity, with 32 distinct PFGE patterns. Most of *A. butzleri* isolates presented multiple antimicrobial resistance, exhibiting four different resistance profiles. All 43 isolates were susceptible to gentamicin and 2.3% were resistant to chloramphenicol, in contrast to twenty four (55.8%) that were resistant to ciprofloxacin. Among 36 selected isolates, 26 strains presented biofilm-forming ability, which was dependent on the atmosphere and initial inoculum density.
Overall, the results showed that *A. butzleri* displays a high genetic diversity, and presents resistance to several antibiotics, which together with its biofilm formation ability may represent a potential hazard for foodborne infections and a considerable risk for human health.

1. Introduction

The genus *Arcobacter* was proposed and included in the family *Campylobacteraceae* in 1991 (Vandamme et al., 1991). The genus currently consists of fifteen recognized species (Vandamme et al., 1992; Donachie et al., 2005; Houf et al., 2005; Collado et al., 2009; Houf et al., 2009; Figueras et al., 2010; Collado et al., 2011; Kim et al., 2010; De Smet et al., 2011; Figueras et al., 2011; Levican et al., 2012), of which *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* have been associated with human and animal illness (Ho et al., 2006).

Arcobacter spp. has been isolated from food, water, food processing and handling facilities, and human samples. *Arcobacter* has commonly been isolated from products of animal origin with the highest prevalence found in chickens, followed by pork and beef (Ho et al., 2006). Indeed, consumption of *Arcobacter*-contaminated food or water is regarded as the most probable transmission route to human and animals (Collado and Figueras, 2011).

Studies have documented the presence of *Arcobacter* spp. in the poultry slaughterhouse environment, along equipments' processing line, including the processing water (Houf et al., 2002a, 2003; Gude et al., 2005), as well as on chicken carcasses at different processing stages of slaughter (Son et al., 2007a), and at retail level (Atabay and Corry, 1997; Atabay et al., 1998). Conflicting results regarding the *Arcobacter* presence in the poultry intestine samples have been reported (Atabay and Corry, 1997; Houf et al., 2002a; Atabay et al., 2006; Van Driessche and Houf, 2007; Ho et al., 2008). Nonetheless, the source of poultry contamination is not so far clear.

The broad distribution of *A. butzleri* has been associated with a high genetic diversity, with multiple genotypes being found in a single location, or even in a single animal (Houf et al., 2003; Son et al., 2006).

Additionally to a vast distribution and variability, resistance to common antimicrobial agents was also observed for *Arcobacter* (Kabeya et al., 2004; Son et al., 2007b; Fera et al., 2003; Vandenberg et al., 2006), constituting a concern among *Campylobacteraceae* family.

In most settings (natural, industrial, or clinical), bacteria are usually found in biofilms rather than in the planktonic state. Biofilms also provide important environmental reservoirs for pathogenic bacteria (Parsek and Singh, 2003), supporting their survival in stressful environments, including food processing facilities and slaughterhouses (Chmielewski and Frank, 2003). Bacteria belonging to the genus *Arcobacter* were isolated from biofilms on the carapace of a live lobster, on the sub-water surfaces of the holding facility (Welsh et al., 2011), and on a multispecies anaerobic biofilm inside a reactor (Fernández et al., 2008). Additionally, *A. butzleri* can attach to several water pipes materials (Assanta et al., 2002) and may reside and proliferate in the

* Corresponding author at: University of Beira Interior, Av. Marquês D' Ávila e Bolama, 6201-001 Covilhã, Portugal. Tel.: +351 275 319 827; fax: +351 275 319 730.
E-mail address: fdomingues@ubi.pt (F.C. Domingues).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.01.003

slaughterhouse environment, due to its ability to form biofilm under chilled conditions (Kjeldgaard et al., 2009).

In this study we have analyzed the distribution, genetic diversity, and virulence associated characteristics, namely antibiotic resistance and biofilm-formation ability, of *Arcobacter* isolated from poultry and environment of a Portuguese slaughterhouse.

2. Materials and methods

2.1. Sample collection

During March 2011, three flocks were sampled at the slaughterhouse: one flock from extensive indoor production system, and two flocks of intensive production systems. The poultry production was characterized as extensive indoor production system when: i) animals stocking density was of 12 birds/m^2 or 25 kg/m^2; ii) the animals had no access to exterior and iii) the birds were not slaughtered before 56 days old. For intensive production systems: i) the animals stocking density was of 14–15 birds/m^2 or up to 42 kg/m^2, ii) with no access to exterior and iii) birds were slaughtered at 5–7 weeks of age. From each flock, ten pre-chilling neck skin and ten caeca were randomly taken. In addition, and also for each flock, 15 mL of water draining off from hanging broiler carcasses (carcass drippings) were collected immediately after the first wash following evisceration. All flocks were slaughtered on the same day at the same plant.

Four samples with approximately 1000 cm^2 were collected with surface collection sponges all along the slaughter line, namely from: i) a ramp before the chilling tunnel at the slaughterhouse; ii) the wall of the evisceration area of extensive indoor production flocks; iii) a conveyor belt after chilling tunnel and iv) a support table.

2.2. Sample processing

Sample processing was performed using the media and *Arcobacter* isolation procedure described by Ho et al. (2008) with slight modifications. Briefly, for the isolation of *Arcobacter*, a representative composite sample by flock was conceived as a pool of 10 samples randomly collected of neck skin or caecum (after removal of feces), totalizing 25 g of sample. Then, 225 mL of *Arcobacter* broth (Oxoid, Hampshire, England) with cefoperazone, amphotericin B, teicoplanin (CAT) selective supplement (Oxoid, Hampshire, England) and novobiocin (Sigma, St Louis, USA) (32 mg/L) (from now on referred to as *Arcobacter* enrichment broth) were added and this mixture was homogenized in a Stomacher® Labblender 400. The surface collection sponges were also homogenized with 225 mL of *Arcobacter* enrichment broth. The carcass drippings were added in a 1:1 proportion with *Arcobacter* enrichment broth. All the homogenates were incubated microaerobically (Anoxomat®, MART Microbiology BV, Drachten, The Netherlands) for 72 h at 30 °C.

From each homogenate, 50 μL was dropped on a cellulose-nitrate membrane filter (0.65 or 0.45 μm) placed on selective blood agar plates (Brain heart infusion agar supplemented with 5% (v/v) defibrinated horse blood (Probiologica, Belas, Portugal) and CAT selective supplement, with or without novobiocin (32 mg/L)). After 1 h incubation at 30 °C, in aerobic atmosphere, the filters were removed and filtrates evenly distribute over the agar surface with a sterile loop of inoculation. These plates were incubated for 48 h at 30 °C under microaerophilic conditions.

The faeces were removed from the caecum and the internal surface was scraped. This caecal content was used in direct plating on selective blood agar plates.

After incubation, at least three colonies suspected to be *Arcobacter* spp. were transferred from selective blood agar plates to blood agar plates (blood agar base Nº 2 (Oxoid, Hampshire, England) supplemented with 5% (v/v) defibrinated horse blood), and incubated for 48 h at 30 °C, under microaerophilic conditions.

2.3. Arcobacter multiplex PCR

For identification of the isolates, an *Arcobacter* species specific multiplex PCR assay was performed using 3 μL of lysed bacteria and the primers SKIR, ARCO, BUTZ, CRY1, and CRY2, previously described (Houf et al., 2000). Amplification products were analyzed by electrophoresis in 1.5% agarose in 0.5x TBE buffer (0.9 M Tris, 0.9 M Boric acid, 0.02 M EDTA pH 8.0). Gels were stained with GelRed and visualized by a UV gel documentation system. *A. butzleri* LMG 6620, *A. cryaerophilus* LMG 10244 and *A. skirrowii* LMG 6621 were used as positive controls.

2.4. Pulsed field gel electrophoresis (PGFE)

Genomic DNA fingerprints of all isolates were determined using PFGE, according to the protocol described in Campynet (http://campynet.vetinst.dk/PFGE.html), but using 50 μg/mL of proteinase K in the preparation of DNA plugs.

Agarose embedded DNA was digested overnight at 25 °C with 20 U of *SmaI*. The isolates with undistinguishable *SmaI* patterns were further digested with 20 U of *SacII*, for 4 h at 37 °C.

The restriction fragments of *Arcobacter* isolates were separated by PFGE on 1.4% agarose gels in 0.5x TBE buffer. Electrophoresis was performed in a CHEF-DR III system (Bio-Rad Laboratories), at initial switch time of 5 s; final switch time of 40 s; angle of 120°; gradient of 6.0 V/cm; at 14 °C; running for 22.5 h for *SmaI* and 26 h for *SacII*. After electrophoresis, gels were stained in 1 μg/mL of ethidium bromide solution for 30 min and then washed with distilled water. Gels were visualized with a UV gel documentation system.

The PFGE patterns of *Arcobacter* were analyzed by InfoQuest FP software (version 5.10) to determine strain relatedness. The optimization setting was 1 and 0.54% and the band position tolerance was 1.8 and 1.0% for restriction with *SmaI* and *SacII*, respectively. Cluster analysis was performed by the Dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA).

2.5. Antibiotic susceptibility

The minimum inhibitory concentration (MIC) was determined for the following nine antibiotics: Ampicillin, Ciprofloxacin, Vancomycin, Trimethoprim, Piperacillin, Cefoperazone, Chloramphenicol, Gentamicin and Amoxicillin.

Arcobacter isolates grown on blood agar plates for 24 h were suspended in 0.85% (w/V) NaCl and adjusted to a turbidity of 0.5 McFarland (~10^8 CFU/mL, determined by colony count). These cell suspensions were diluted 1:100 on Mueller-Hinton (MH) broth to obtain a density of about 10^6 CFU/mL. Serial two-fold dilutions of each antibiotic were prepared in a 96-well plate (50 μL per well) and inoculated with 50 μL of the bacterial suspension. The plates were incubated at 37 °C for 48 h under microaerophilic conditions. The MIC was confirmed spectrophotometrically at 620 nm, using a cut-off of 0.05. Each experiment was repeated at least three times at each test concentration and the modal MIC values were selected.

To date, no recommendation of breakpoint values for *Arcobacter* is available. The breakpoints used for ampicillin, ciprofloxacin, chloramphenicol, gentamicin and amoxicillin were those of *Campylobacter* species following the National Antimicrobial Resistance Monitoring System criteria (CDC, 2006, 2010). Interpretative criteria for piperacillin, vancomycin, trimethoprim and cefoperazone followed the Clinical and Laboratory Standards Institute protocol M100 (CLSI, 2005).

2.6. Biofilm formation assay and biofilm quantification

The ability of *Arcobacter* strains to form biofilms was assayed using a previously described method, with slight modifications (McLennan et al., 2008; Gaynor et al., 2007). Briefly, cells were grown overnight in MH broth at 37 °C and diluted to an optical density (OD) at 620 nm