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Prevalence and antimicrobial susceptibility of *Acinetobacter* spp. isolated from meat

Ana Carvalheira, Rocio Casquete, Joana Silva, Paula Teixeira *

CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

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ABSTRACT

The prevalence and antibiotic resistance of *Acinetobacter* spp. from fifty samples of meat (chicken, turkey, beef and pork) were evaluated. *Acinetobacter* spp. was recovered from all samples and the clonal relatedness of 223 isolates identified to belong to the genus *Acinetobacter* was established by PFGE. A high genetic diversity was observed and 166 isolates from different samples, 141 representing different PFGE profiles, were further identified to the species level by *rpoB* gene sequencing. Thirteen distinct *Acinetobacter* species were identified among 156 isolates. The remaining ten isolates may represent three putatively novel species since *rpoB* sequence homologies with type strains of all available described *Acinetobacter* species, were <95%.

The most common species was *Acinetobacter guillouiae* with a prevalence of 34.9%. However 18.7% of the strains belong to the *Acinetobacter baumannii* group ($n = 31$) which include the species *Acinetobacter baumannii* ($n = 7$), *Acinetobacter pittii* ($n = 12$), *Acinetobacter seifertii* ($n = 8$) and *Acinetobacter nosocomialis* ($n = 4$) that are the species most frequently associated with nosocomial infections worldwide.

In general, strains were resistant to some of the antimicrobials most frequently used to treat *Acinetobacter* infections such as piperacillin-tazobactam (64.9% of strains resistant), ceftazidime (43.5%), ciprofloxacin (42.9%), as well as to colistin (41.7%) and polymyxin B (35.1%), the last-resort drugs to treat infections caused by multidrug-resistant *Acinetobacter*. The percentage of resistant strains to trimethoprim-sulfamethoxazole, tetracycline, aminoglycosides (amikacin and tobramycin) and ampicillin-sulbactam was >10% (23.2%, 23.2%, 14.3%, 12.5%, 12.5%, respectively). However, resistances to meropenem, imipenem and minocycline were only sporadically observed (8.3%, 1.2% and 1.2%, respectively).

Overall, 51.2% of the strains were considered as multidrug-resistant (MDR) and 9.6% as extensively drug-resistant (XDR). The prevalence of MDR strains within the *A. baumannii* group (38.7%) was lower than the prevalence within the others species identified (54.1%). Therefore, food of animal origin may be a vehicle of spread *Acinetobacter* strains resistant to several antibiotics in the community and in the hospital setting environment. This may led to nosocomial and community-acquired infections in susceptible individuals.

1. Introduction

Members of the genus *Acinetobacter* are strictly aerobic non-fermenting Gram-negative cocco-bacilli currently including 39 validly named species (Euzéby, 2016). *Acinetobacter baumannii* group i.e. *Acinetobacter baumannii, A. pittii, A. nosocomialis* and *A. seifertii*, includes the species most often associated with nosocomial infections worldwide (Dijkshoorn et al., 2007; Nemec et al., 2015; Peleg et al., 2008). Moreover, these opportunistic pathogens have been implicated in community-acquired infections (Chang et al., 2000; Falagas et al., 2007; Falagas and Rafailidis, 2007; Kang et al., 2012). The ubiquity of *A. baumannii* in nature has been considered a widespread misconception due to difficulties in unequivocal species identification (Dijkshoorn et al., 2007; Eveillard et al., 2013; Peleg et al., 2008; Towner, 2009; Visca et al., 2011). Identification by molecular methods such *rpoB* gene sequencing improved the identification across the genus (Gundi et al., 2009; La Scola et al., 2006). Additionally, with the use of more reliable identification methods, other species outside the *A. baumannii* group, such as *A. lwoffii*, *A.ursingii*, *A. johnsonii* and *A. parvus* have also been implicated in nosocomial infections, and may represent emerging pathogens (Turton et al., 2010).

Owing to its remarkable ability to resist almost all available antimicrobial agents, *Acinetobacter* infections are difficult to treat. Indeed, multidrug-resistant or even pan-drug resistant isolates are increasing alarmingly in the hospital environment (Coyne et al., 2010; Dijkshoorn et al., 2007; Kempf and Rolain, 2012; Peleg et al., 2008; Zarrilli et al., 2013). This characteristic, associated with the ability of

* Corresponding author.
E-mail address: pcteixeira@porto.ucp.pt (P. Teixeira).

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these organisms to survive under diverse environmental conditions (Bergogne-Bérézin and Towner, 1996; Fournier and Richet, 2006; Jawad et al., 1996), facilitates their survival and spread. Therefore, it is important to identify and monitor the possible sources and routes of transmission to community and hospital settings. Worldwide, the spread of antibiotic resistant bacteria through food is considered a major public health concern since the food chain has an important role in the dissemination of some important human pathogens (Perreten, 2005; Seiffert et al., 2013a; Seiffert et al., 2013b; Verraes et al., 2013). Furthermore, the widespread use of antibiotics in food-producing animals has been linked to the emergence and dissemination of resistant bacteria (Phillips et al., 2004), which can further be spread to community and hospital settings through food.

The prevalence and antimicrobial resistance of *Acinetobacter* species isolated from human clinical isolates has been reported. However, studies about their prevalence in meat samples are limited. *Acinetobacter* spp. has been isolated from veterinary clinical specimens of food animals in the UK (Hamouda et al., 2008), Lebanon (Rafei et al., 2015), France (Poirel et al., 2012) and China (Wang et al., 2012; Zhang et al., 2013); in some cases, carbapenemase-producing isolates were described (Poirel et al., 2012; Wang et al., 2012; Zhang et al., 2013). Nevertheless, only a few studies have reported the presence *Acinetobacter* spp. in raw meat (Hamouda et al., 2008; Houang et al., 2001; Lupo et al., 2014; Rafei et al., 2015; Saha and Chopade, 2001); whereas the antimicrobial susceptibility of the isolates was not determined (Houang et al., 2001; Saha and Chopade, 2001), others refer to the prevalence of the specie *A. baumannii* (Hamouda et al., 2008; Lupo et al., 2014).

The objective of this study was to evaluate the prevalence and diversity of *Acinetobacter* spp. in meat samples, as well as their antibiotic resistance.

2. Materials and methods

2.1. Samples

Between October 2013 and September 2014, fifty meat samples purchased from five supermarkets located in Porto region (Portugal), belonging to four convenience store groups were analysed. These included chicken breast ($n = 14$), turkey breast ($n = 12$), pork steaks ($n = 12$) and beef steaks ($n = 12$) samples, all without skin and bones. From each store, no more than one sample of each meat type was collected on the same day.

2.2. Prevalence of Acinetobacter spp. in meat samples

2.2.1. Isolation method

The isolation of *Acinetobacter* spp. from meat samples was done according to Carvalheira et al. (2016). As the likely level of contamination by these organisms was largely unknown, the sampling method used for the detection of salmonellae was used: 25 g composite samples in enrichment as recommended by the EC regulation No. 2073/2005 for meat products (EC, 2005). Briefly, 25 g of each product were added to 225 mL of Dijkshoorn enrichment medium and homogenized in a Stomacher (Interscience, St Nom la Bretêche, France) for 2 min. After incubation for 24 h at 30 °C in an orbital shaker (150 rpm; New Brunswick Scientific Co. Inc., New Jersey, USA) one loopful of enrichment broth was plated by streaking on CHROMagar™ Acinetobacter (CHROMagar, Paris, France), incubated at 30 °C for 24–48 h, and examined for the growth of typical red colonies of *Acinetobacter* spp.

2.3. Identification of Acinetobacter spp.

2.3.1. Confirmation of presumptive colonies

One typical colony representative of each type of morphology and shape was further sub-cultured on TSA and characterized based on phenotypic tests: Gram-stain, catalase and oxidase tests. Gram-negative coccobacilli, oxidase-negative, and catalase-positive isolates were presumptively identified to the genus *Acinetobacter*. Confirmation to the genus level was performed by the presence of *Acinetobacter* spp. 16S rRNA signature, as previously described by Vanbroekhoven et al. (2004).

2.3.2. Selection of isolates for identification to the species level

In order to avoid some repetitive isolates from each sample all isolates belonging to the genus *Acinetobacter* were genotyped by Pulsed-field Gel Electrophoresis (PFGE). From each sample, only one isolate of each PFGE profile was selected for further analysis. PFGE was performed according to Chang et al. (2013), using the restriction enzyme *Asc*I (New England Biolabs, Ipswich, MA), with minor modifications: the optical density of bacterial cell suspensions used was adjusted to 1.3 at 600 nm and the minimal amount of *Asc*I was 5 U. The PFGE was run in a CHEF Mapper XA (Bio-Rad Laboratories, Hercules, CA), with pulses ranging from 4 to 40 s at 14 °C with 6 V/cm for 21 h. The band patterns were analysed by GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) and the cluster analysis of PFGE profiles was done by the unweighted-pair group method with average linkages (UPGMA), using the Dice coefficient to generate a dendrogram describing the relationship among PFGE profiles. A band position tolerance of 1.0 was selected and the classification of isolates into different *Asc*I patterns was visually validated.

2.3.3. Species identification

Species identification was performed for representative isolates of each PFGE profile based on the analysis of the sequence of the region Z1–Z2 of the gene for RNA polymerase beta-subunit (*rpoB*) according to La Scola et al. (2006). The partial sequences of *rpoB* (902 bp) were amplified with the primers (Ac696F and Ac1598R) and in conditions described by La Scola et al. (2006). PCR products were purified, using the GRS PCR & Gel Band Purification Kit (Grisp, Porto, Portugal), according to the supplier's instructions, and the DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). The partial *rpoB* nucleotide sequences were edited manually, using the software BioEdit 7.1.3.0 (Hall, 1999). Similarity between the *rpoB* nucleotide sequences of the tested isolates and the *rpoB* sequences of the type strains of all *Acinetobacter* species available in the GenBank database (http://www.ncbi.nlm.nih.gov) was calculated by using the MEGA6 software (Tamura et al., 2013). Isolates showing at least 95% *rpoB* gene sequence similarity with a type strain were identified as specific *Acinetobacter* species (Krizova et al., 2014; La Scola et al., 2006).

2.4. Antimicrobial resistance

Antimicrobial resistance of representative isolates of each PFGE profile, including strains with the same PFGE pattern when recovered from different meat samples, was investigated according to the standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2012) using Mueller Hinton agar (MHA, BioMérieux, Marcy-l'Étoile, France). The disk diffusion method was used to test twelve antibiotics: piperacillin (PIP, 100 μg), piperacillin-tazobactam (TZP 100/10 μg), ampicillin-sulbactam (SAM 10/10 μg), ceftazidime (CAZ, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), amikacin (AK, 30 μg), tobramycin (TOB, 10 μg), tetracycline (TE, 30 μg), minocycline (MI, 30 μg), ciprofloxacin (CIP, 5 μg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75 μg) (all from Oxoid, Hants, UK). Susceptibility to colistin (CL) and polymyxin B (PB) was evaluated by the agar dilution method with antibiotic concentrations ranging from 1 to 8 μg/mL. Cultures were incubated for 24 h at 35 °C and *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* DSM 1117 were used as quality control strains. The inhibition zones were measured and the strains were categorized as susceptible or resistant according to CLSI interpretive criteria (CLSI, 2012).