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Short communication

Pathogenic *Acinetobacter* species including the novel *Acinetobacter dijkshoorniae* recovered from market meat in Peru

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ABSTRACT

Species of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex are important human pathogens which can be recovered from animals and food, potential sources for their dissemination. The aim of the present study was to characterise the *Acinetobacter* isolates recovered from market meat samples in Peru. From July through August 2012, 138 meat samples from six traditional markets in Lima were cultured in Lysogeny and Selenite broths followed by screening of Gram-negative bacteria in selective media. Bacterial isolates were identified by MALDI-TOF MS and DNA-based methods and assessed for their clonal relatedness and antimicrobial susceptibility.

Twelve *Acinetobacter* isolates were recovered from calf samples. All but one strain were identified as members of the clinically-relevant *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex: 9 strains as *Acinetobacter pittii*, 1 strain as *A. baumannii*, and 1 strain as the recently described novel species *A. dijkshoorniae*.

The remaining strain could not be identified at the species level unambiguously but all studies suggested close relatedness to *A. bereziniae*. All isolates were well susceptible to antibiotics. Based on macrorestriction analysis, six isolates were further selected and some of them were associated with novel MLST profiles.

The presence of pathogenic *Acinetobacter* species in human consumption meat might pose a risk to public health as potential reservoirs for their further spread into the human population. Nevertheless, the *Acinetobacter* isolates from meat found in this study were not multidrug resistant and their prevalence was low. To our knowledge, this is also the first time that the *A. dijkshoorniae* species is reported in Peru.

1. Introduction

The genus *Acinetobacter* currently comprises 60 validly published species names including four pairs of synonyms and a number of tentative species without standing in nomenclature (https://apps.szu.cz/anemec/Classification.pdf, last accessed April 2019), some of them environmental and some considered human pathogens. Among the latter, species within the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex are of particular clinical relevance, often associated with nosocomial infections (Cosgaya et al., 2016; Nemec et al., 2015; Roca et al., 2012). *Acinetobacter baumannii* is clearly the most prevalent pathogen of the ACB complex, probably due to its inherent ability to persist and survive in the hospital environment as well as to acquire resistance to multiple antimicrobial drugs and disinfectants (Lee et al., 2017). Despite the clinical relevance of members of the ACB complex, pathogenic *Acinetobacter* spp. have also been reported from food and food-producing animals, which might constitute an overlooked reservoir and source of bacterial pathogens to the human population (Carvalheira et al., 2017a; Carvalheira et al., 2017b; Hamouda et al., 2011; Lupo et al., 2014).

Abbreviations: ACB complex, *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex; CC, clonal complex; MALDI-TOF MS, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; MIC, minimum inhibitory concentration; MLSA, multilocus sequence analysis; MLST, multilocus sequence typing; PFGE, pulse-field gel electrophoresis; ST, sequence type

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The aim of the present study was to analyse the phenotypic and genotypic characteristics of *Acinetobacter* spp. recovered from market meat samples in Peru as well as to characterise their clonal relatedness.

2. Material and methods

2.1. Samples and isolation method

From July through August 2012, 138 meat samples from poultry ($n = 64$), swine ($n = 30$) and beef ($n = 44$) were obtained by random sampling from six traditional markets scattered throughout the city of Lima, Peru. Meat samples were transported in sterile bags to the laboratory, homogenised in a paddle blender (Stomacher® 400 circulator, Seward, UK) and 2 g of each were used to enrich the bacterial burden in overnight cultures with Lysogeny and Selenite broths. Liquid cultures were plated in different agar media such as Xylose Lysine Deoxycholate agar, Salmonella-Shigella agar, MacConkey agar and Hektoen agar (all from Oxoid, Basingstoke, UK) to select for different Enterobacteria (Ruiz-Roldan et al., 2018). Specific enrichment media or pre-enrichment steps for *Acinetobacter* were not used as this study was initially designed to capture *Enterobacterales* only. Further growth of bacterial isolates was performed in Columbia sheep blood agar plates (Becton Dickinson GmbH, Heidelberg, Germany) at 37 °C.

2.2. Bacterial identification and typing

2.2.1. Identification by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

Bacterial identification for all grown colonies was performed by MALDI-TOF MS in a Microflex LT benchtop instrument (Bruker Daltonics, Bremen, Germany). Spectra were analysed with the MALDI BioTyper software (version 3.1; Bruker Daltonics) using the pre-processing and BioTyper main spectrum (MSP) identification standard methods (mass range 2000–20,000 *m/z*) against the default Bruker database (V.8.0.0.0). Accuracy of the identification was determined by a logarithmic score value resulting from the alignment of peaks to the best matching reference spectrum (Espinal et al., 2012).

2.2.2. Selection of isolates for identification with molecular methods

All isolates belonging to the *Acinetobacter* genus were analysed by Pulsed-field Gel Electrophoresis (PFGE) as described previously (Uwingabiye et al., 2017), using genomic digestions with the *Apa*I restriction enzyme. Electrophoresis was performed in 1% InCert™ Agarose (Lonza, Rockland, ME, USA) and 0.5× TBE Buffer (pH 8.0) containing 0.02 g of thiourea using either a CHEF-DR III system (Bio-Rad Laboratories, Marnes-la-Coquette, France) or a CHEF-Mapper™ apparatus (Bio-Rad Laboratories) at 6 V/cm2 with switch times ranging from 5 s to 35 s at an angle of 120°, at temperature of 14 °C, for 20 h.

Molecular patterns were analysed with the InfoQuest™ FP v.5.4 software (Bio-Rad Laboratories) and the unweighted pair group method with arithmetic mean to create dendrograms based on Dice's similarity coefficient. Using bandwidth tolerance and optimisation values set at 1 and 1%, respectively, isolates were considered to belong to the same PFGE cluster (pulsotype) if their Dice similarity index was ≥85% (Durmaz et al., 2009).

2.2.3. Identification to the species level and sequence typing

For selected isolates, the partial sequences of the *rpoB* gene (zones 1 and 2) were amplified by PCR and sequenced using primers Ac696F and Ac1598R (902 bp), according to La Scola et al. (2006). Likewise, the partial sequences of *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*, the seven housekeeping genes from the Pasteur multilocus sequence typing (MLST) scheme, were also amplified and sequenced according to Diancourt et al. (2010). Partial *rpoB* sequences and the concatenated partial sequences of MLST genes were used to analyse sequence similarity between the tested isolates and reference strains belonging to the ACB complex as well as other *Acinetobacter* species that were retrieved from public repositories (Cosgaya et al., 2016). Sequence alignment was done with Clustal Ω and phylogenetic trees were constructed using the neighbour-joining method, with genetic distances computed by Kimura's two-parameter model (Kimura, 1980; Sievers et al., 2011). The allele sequences of housekeeping genes were submitted to the *Acinetobacter* MLST database (http://pubmlst.org/abaumannii/) to identify the corresponding sequence types (STs).

2.3. Antibiotic susceptibility testing

Antibiotic susceptibility testing of all isolates of *Acinetobacter* was assessed by gradient diffusion (E test, AB bioMérieux, Solna, Sweden) on Mueller–Hinton agar plates (bioMérieux, Marcy-l'Étoile, France) in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for the following antimicrobials: amikacin, tobramycin, kanamycin, ciprofloxacin, levofloxacin, cefepime, ceftazidime, meropenem, imipenem, chloramphenicol and tigecycline. The minimum inhibitory concentrations (MICs) were interpreted according to EUCAST clinical breakpoints and expert rules for *Acinetobacter* (Version 8.0, January 2018), except for tigecycline, which was interpreted using the EUCAST breakpoints and rules for *Enterobacteriaceae* (Version 8.0, January 2018) (EUCAST, 2018). Susceptibility to colistin was assessed by broth microdilution as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group (EUCAST, 2016). *Escherichia coli* ATCC 25922 and *A. baumannii* ATCC 19606 were used as quality control strains.

2.4. Nucleotide sequence accession numbers

The partial *rpoB* sequences of *Acinetobacter* strains were submitted to GenBank with accession numbers MK382383 (APT-6), MK382384 (APT-1), MK382385 (APT-5), MK382386 (APT-7B), MK382387 (APT-8), MK382388 (APT-7T).

3. Results and discussion

3.1. Identification of Acinetobacter species and clonal relatedness of all isolates

Twelve isolates of the genus *Acinetobacter* were recovered on MacConkey agar plates from the meat samples of five different calves obtained in two independent markets in Lima, Peru.

Species identification of the 12 isolates was initially performed by MALDI-TOF MS by comparing the spectra profiles against the updated Bruker taxonomy database (V.8.0.0.0) which included custom reference spectra for *Acinetobacter dijkshoorniae* and *Acinetobacter seifertii*, the novel members of the ACB complex (Marí-Almirall et al., 2017).

Three isolates were identified as *A. baumannii*, *Acinetobacter bereziniae* and *A. dijkshoorniae*, respectively, and the remaining nine isolates were identified as *Acinetobacter pittii*. Of note, the log score values for the identification of five out of the nine *A. pittii* isolates ranged between 1.9 and 2.01, which constituted a poor probable species identification, according to the manufacturer's specifications.

The PFGE analysis of *Apa*I-digested genomic DNA revealed five distinct fingerprints (A-E) (Table 1). *Acinetobacter pittii* isolates recovered from meat samples from the same calf shared identical fingerprints (B and D), plus one *A. pittii* isolate that was isolated from meat derived from a third calf (together with *A. bereziniae*) and also presented the D fingerprint.

Six isolates were selected for further characterisation, one representative isolate from each fingerprint plus the *A. pittii* isolate that derived from a different calf (Table 1). Identification at the species level for these isolates was re-evaluated using two different molecular methods; sequencing of the partial *rpoB* gene sequences (La Scola et al., 2006), and by multilocus sequence analysis (MLSA) of the concatenated

Table 1
Characteristics of the *Acinetobacter* spp. isolates recovered from market meat samples in Lima, Peru.

Rows display the genotypic and phenotypic information of isolates under the same PFGE cluster and/or origin of the samples. Data was obtained from a representative isolate each. n, number of isolates; Calf, animal from which they were recovered; Area, the location of the markets in Lima; Isolate, designation of the representative isolate for each pulsotype; Species, identification to the species level; ST, sequence type; MIC, minimum inhibitory concentration; AMK, amikacin; TOB, tobramycin; KAN, kanamycin; CIP, ciprofloxacin; LVX, levofloxacin; COL, colistin; FEP, cefepime; CAZ, ceftazidime; MEM, meropenem; IPM, imipenem; CHL, chloramphenicol; TGC, tigecycline.

PFGE	n	Calf	Area	Isolate	Species	ST	MIC (μg/mL)											
							AMK	TOB	KAN	CIP	LVX	COL	FEP	CAZ	MEM	IPM	CHL	TGC
A	1	I	North	APT-1	*A. dijkshoorniae*	1256	2	0.19	1.5	0.5	0.38	0.38	4	4	0.25	0.25	256	0.5
B	4	II	North	APT-5	*A. pittii*	1257	3	0.19	1.5	0.25	0.38	0.38	4	6	0.38	0.38	96	0.38
C	1	III	Centre	APT-6	*A. baumannii*	273	2	0.25	1	0.25	0.25	0.5	2	3	0.25	0.25	96	0.38
D	1	IV	Centre	APT-7B	*A. pittii*	312	1.5	0.125	1	0.25	0.25	0.125	3	3	0.19	0.25	4	0.38
E	1	IV	Centre	APT-7 T	*A. bereziniae*-like	1258	0.75	0.047	0.047	0.25	0.25	1.5	1	4	0.38	0.25	16	0.38
D	4	V	Centre	APT-8	*A. pittii*	312	1.5	0.125	1	0.38	0.25	0.125	3	3	0.25	0.25	8	0.19

partial sequences of all house-keeping genes used for MLST (Diancourt et al., 2010). Molecular methods confirmed the identification of *A. baumannii*, *A. dijkshoorniae* and *A. pittii* isolates from fingerprints A-C. Isolates within fingerprint D comprised those isolates with low MALDI-TOF log score values for *A. pittii* and showed the highest sequence similarity to *A. pittii* (97.3–98.1% and 98.4–99.0% for *rpoB* and MLSA, respectively). This isolates were, therefore, considered as *A. pittii*, even though the *A. pittii* group often embraces strains that are somehow related to each other but do not always meet the technical requirements used for species circumscription. Finally, the isolate corresponding to fingerprint E, designated as *A. bereziniae* by MALDI-TOF MS, clustered together with *Acinetobacter bereziniae* isolates with 96.4–96.9% and 96.6–96.7% similarity for *rpoB* and MLSA, respectively. These values are slightly lower than similarities usually seen between strains of a single species (> 97.5%; Nemec et al., 2015). Accordingly, this isolate was designated as *A. bereziniae*-like although whole-genome sequencing could aid to clarify the taxonomic position of this isolate. Nevertheless, we acknowledge that additional isolates with similar characteristics are needed before proposing the existence of new taxa.

3.2. Susceptibility to antimicrobial agents and epidemiological typing

Antimicrobial susceptibility testing of the selected isolates by gradient diffusion and broth microdilution (colistin) showed low MIC values to all the antimicrobial agents tested except for chloramphenicol, to which MIC values below 8 mg/L were only observed in both *A. pittii* isolates representative from fingerprint D (Table 1). Of note, all isolates showed baseline MIC values between 3 and 6 μg/mL to ceftazidime and between 1 and 4 μg/mL to cefepime, which might be attributed to the intrinsic resistance of *Acinetobacter* spp. to cephalosporins. These results are in agreement with those of Rafei et al. (2015), that showed susceptibility to most antimicrobials in *Acinetobacter* spp. recovered from soil, animals and food products, with only a few carbapenem-resistant isolates that were associated to the carriage of acquired OXA-type carbapenemases, and also with those from Hamouda et al. (2011) that reported high MIC values to chloramphenicol as well as similar baseline MIC values to ceftazidime. In contrast, Carvalheira et al. (2017a) showed higher prevalence of resistance to cephalosporins and quinolones in *Acinetobacter* spp. isolated from meat (43.5% and 42.9%, respectively), and surprisingly high prevalence of resistance to colistin and polymyxin B (41.7% and 35.1%, respectively), although MIC values were not provided and a wider variety of *Acinetobacter* species were reported.

MLST studies identified the *A. baumannii* isolate as belonging to ST273, which had been previously reported in 2000 from a hospitalised patient in Spain and had also been identified from a calf in Switzerland in 2013 (Lupo et al., 2014). ST273 is clustered into clonal complex (CC) CC33, which contains STs related to clinical isolates. The *A. pittii* isolates from fingerprint D were assigned to ST312, an ST previously described from a patient in Belgium in 2009 but to our knowledge, never reported in animals and not included in any CC. The *A. dijkshoorniae* and *A. bereziniae*-like isolates and the *A. pittii* isolate from fingerprint B presented novel MLST alleles and were assigned new sequence types (ST1256, ST1258 and ST1257, respectively) by the curators at https://pubmlst.org/. None of them clustered in any CC either.

3.3. Final remarks

The identification of *Acinetobacter* isolates belonging to several species of the ACB group in raw meat samples is of particular concern, since these species are usually associated with the clinical setting. Previous studies had already reported the presence of species belonging to this group from diverse meat samples, including poultry, swine and beef, although members of the ACB group were not necessarily the most prevalent species (Carvalheira et al., 2017a). In our study, *Acinetobacter* isolates were only recovered from beef samples and the majority of isolates were identified as *A. pittii*. Interestingly, the authors from a study in Lebanon (Rafei et al., 2015) also reported the presence of *A. baumannii*, *A. pittii* and *A. bereziniae* in cow meat samples, with a prevalence similar to that observed in our investigation (28% and 27%, respectively) and *A. pittii* was again the predominant *Acinetobacter* species. Multidrug resistant *Acinetobacter* spp. from the ACB complex currently represent a serious threat to public health but, despite some of the *Acinetobacter* isolates recovered in the present study showing genetic links to clinical isolates, all isolates were susceptible to clinically relevant antibiotics and their overall prevalence was low (8.6%). Nevertheless, contaminated meat should not be neglected as a source for the transmission of *Acinetobacter* spp. into domestic and hospital settings where it may also contribute to the evolution of clinical lineages which, ultimately, might accumulate resistance genes.

It is also worth mentioning that the actual burden of *Acinetobacter* spp. from market meat samples may be much higher than what was shown in our study, since specific enrichment media or pre-enrichment steps for *Acinetobacter* were not used.

To our best knowledge, we also report here the first identification of the recently described *A. dijkshoorniae* in meat samples of animal origin as well as its first identification in Peru.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Declaration of Competing Interest

None.

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