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.

Keywords: food-producing animals; antimicrobial resistance; epidemiology; taxonomy

26

27 **Abbreviations**: ACB complex, *Acinetobacter calcoaceticus–Acinetobacter*

28 *baumannii* complex; CC, clonal complex; MALDI-TOF MS, matrix-assisted laser

29 desorption ionisation time-of-flight mass spectrometry; MIC, minimum inhibitory

30 concentration; MLSA, multilocus sequence analysis; MLST, multilocus sequence

31 typing; PFGE, pulse-field gel electrophoresis; ST, sequence type.

32 **1. Introduction**

33 The genus *Acinetobacter* currently comprises 60 validly published species names
34 including four pairs of synonyms and a number of tentative species without standing
35 in nomenclature (https://apps.szu.cz/anemec/Classification.pdf, last accessed April
36 2019), some of them environmental and some considered human pathogens. Among
37 the latter, species within the *Acinetobacter calcoaceticus-Acinetobacter baumannii*
38 (ACB) complex are of particular clinical relevance, often associated with nosocomial
39 infections (Cosgaya et al., 2016; Nemec et al., 2015; Roca et al., 2012). *Acinetobacter*
40 *baumannii* is clearly the most prevalent pathogen of the ACB complex, probably due
41 to its inherent ability to persist and survive in the hospital environment as well as to
42 acquire resistance to multiple antimicrobial drugs and disinfectants (Lee et al., 2017).
43 Despite the clinical relevance of members of the ACB complex, pathogenic
44 *Acinetobacter* spp. have also been reported from food and food-producing animals,
45 which might constitute an overlooked reservoir and source of bacterial pathogens to
46 the human population (Carvalheira et al., 2017a; Carvalheira et al., 2017b; Hamouda
47 et al., 2011; Lupo et al., 2014).
48 The aim of the present study was to analyse the phenotypic and genotypic
49 characteristics of *Acinetobacter* spp. recovered from market meat samples in Peru as
50 well as to characterise their clonal relatedness.
51

52 **2. Material and methods**

53 *2.1. Samples and isolation method*

54 From July through August 2012, 138 meat samples from poultry (n=64), swine
55 (n=30) and beef (n=44) were obtained by random sampling from six traditional
56 markets scattered throughout the city of Lima, Peru. Meat samples were transported

57 in sterile bags to the laboratory, homogenised in a paddle blender (Stomacher® 400
58 circulator, Seward, UK) and 2 g of each were used to enrich the bacterial burden in
59 overnight cultures with Lysogeny and Selenite broths. Liquid cultures were plated in
60 different agar media such as Xylose Lysine Deoxycholate agar, Salmonella-Shigella
61 agar, MacConkey agar and Hektoen agar (all from Oxoid, Basingstoke, UK) to select
62 for different Enterobacteria (Ruiz-Roldan et al., 2018). Specific enrichment media or
63 pre-enrichment steps for *Acinetobacter* were not used as this study was initially
64 designed to capture *Enterobacterales* only. Further growth of bacterial isolates was
65 performed in Columbia sheep blood agar plates (Becton Dickinson GmbH,
66 Heidelberg, Germany) at 37ºC.

67 *2.2. Bacterial identification and typing*

68 *2.2.1. Identification by matrix-assisted laser desorption/ionisation time-of-flight mass*
69 *spectrometry (MALDI-TOF MS)*

70 Bacterial identification for all grown colonies was performed by MALDI-TOF MS in
71 a Microflex LT benchtop instrument (Bruker Daltonics, Bremen, Germany). Spectra
72 were analysed with the MALDI BioTyper software (version 3.1; Bruker Daltonics)
73 using the pre-processing and BioTyper main spectrum (MSP) identification standard
74 methods (mass range 2,000-20,000 m/z) against the default Bruker database
75 (V.8.0.0.0). Accuracy of the identification was determined by a logarithmic score
76 value resulting from the alignment of peaks to the best matching reference spectrum
77 (Espinal et al., 2012).

78 *2.2.2. Selection of isolates for identification with molecular methods*

79 All isolates belonging to the *Acinetobacter* genus were analysed by Pulsed-field Gel
80 Electrophoresis (PFGE) as described previously (Uwingabiye et al., 2017), using
81 genomic digestions with the ApaI restriction enzyme. Electrophoresis was performed

82 in 1% InCert^{TM} Agarose (Lonza, Rockland, ME, USA) and 0.5X TBE Buffer (pH 8.0)
83 containing 0.02 g of thiourea using either a CHEF-DR III system (Bio-Rad
84 Laboratories, Marnes-la-Coquette, France) or a CHEF-Mapper^{TM} apparatus (Bio-Rad
85 Laboratories) at 6 V/cm2 with switch times ranging from 5 s to 35 s at an angle of
86 120°, at temperature of 14 °C, for 20 h.
87 Molecular patterns were analysed with the InfoQuest^{TM} FP v.5.4 software (Bio-Rad
88 Laboratories) and the unweighted pair group method with arithmetic mean to create
89 dendrograms based on Dice's similarity coefficient. Using bandwidth tolerance and
90 optimisation values set at 1 and 1%, respectively, isolates were considered to belong
91 to the same PFGE cluster (pulsotype) if their Dice similarity index was ≥85%
92 (Durmaz et al., 2009).
93 *2.2.3. Identification to the species level and sequence typing*
94 For selected isolates, the partial sequences of the *rpoB* gene (zones 1 and 2) were
95 amplified by PCR and sequenced using primers Ac696F and Ac1598R (902 bp),
96 according to La Scola et al. (2006). Likewise, the partial sequences of *cpn60*, *fusA*,
97 *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*, the seven housekeeping genes from the Pasteur
98 multilocus sequence typing (MLST) scheme, were also amplified and sequenced
99 according to Diancourt et al. (2010). Partial *rpoB* sequences and the concatenated
100 partial sequences of MLST genes were used to analyse sequence similarity between
101 the tested isolates and reference strains belonging to the ACB complex as well as
102 other *Acinetobacter* species that were retrieved from public repositories (Cosgaya et
103 al., 2016). Sequence alignment was done with Clustal Ω and phylogenetic trees were
104 constructed using the neighbour-joining method, with genetic distances computed by
105 Kimura's two-parameter model (Kimura, 1980; Sievers et al., 2011). The allele
106 sequences of housekeeping genes were submitted to the *Acinetobacter* MLST

107 database (http://pubmlst.org/abaumannii/) to identify the corresponding sequence
108 types (STs).

109 *2.3. Antibiotic susceptibility testing*

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

124 *2.4 Nucleotide sequence accession numbers*

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

128

129 **3. Results and discussion**

130 *3.1. Identification of Acinetobacter species and clonal relatedness of all isolates*

131 Twelve isolates of the genus *Acinetobacter* were recovered on MacConkey agar plates
132 from the meat samples of five different calves obtained in two independent markets in
133 Lima, Peru.
134 Species identification of the 12 isolates was initially performed by MALDI-TOF MS
135 by comparing the spectra profiles against the updated Bruker taxonomy database
136 (V.8.0.0.0) which included custom reference spectra for *Acinetobacter dijkshoorniae*
137 and *Acinetobacter seifertii*, the novel members of the ACB complex (Marí-Almirall et
138 al., 2017).
139 Three isolates were identified as *A. baumannii*, *Acinetobacter bereziniae* and *A.*
140 *dijkshoorniae*, respectively, and the remaining nine isolates were identified as
141 *Acinetobacter pittii.* Of note, the log score values for the identification of five out of
142 the nine *A. pittii* isolates ranged between 1.9 and 2.01, which constituted a poor
143 probable species identification, according to the manufacturer's specifications.
144 The PFGE analysis of ApaI-digested genomic DNA revealed five distinct fingerprints
145 (A-E) (Table 1). *Acinetobacter pittii* isolates recovered from meat samples from the
146 same calf shared identical fingerprints (B and D), plus one *A. pittii* isolate that was
147 isolated from meat derived from a third calf (together with *A. bereziniae*) and also
148 presented the D fingerprint.
149 Six isolates were selected for further characterisation, one representative isolate from
150 each fingerprint plus the *A. pittii* isolate that derived from a different calf (Table 1).
151 Identification at the species level for these isolates was re-evaluated using two
152 different molecular methods; sequencing of the partial *rpoB* gene sequences (La Scola
153 et al., 2006), and by multilocus sequence analysis (MLSA) of the concatenated partial
154 sequences of all house-keeping genes used for MLST (Diancourt et al., 2010).
155 Molecular methods confirmed the identification of *A. baumannii*, *A. dijkshoorniae*

156 and *A. pittii* isolates from fingerprints A-C. Isolates within fingerprint D comprised
157 those isolates with low MALDI-TOF log score values for *A. pittii* and showed the
158 highest sequence similarity to *A. pittii* (97.3-98.1% and 98.4-99.0% for *rpoB* and
159 MLSA, respectively). This isolates were, therefore, considered as *A. pittii*, even
160 though the *A. pittii* group often embraces strains that are somehow related to each
161 other but do not always meet the technical requirements used for species
162 circumscription. Finally, the isolate corresponding to fingerprint E, designated as *A.*
163 *bereziniae* by MALDI-TOF MS, clustered together with *Acinetobacter bereziniae*
164 isolates with 96.4-96.9% and 96.6-96.7% similarity for *rpoB* and MLSA,
165 respectively. These values are slightly lower than similarities usually seen between
166 strains of a single species (>97.5%; Nemec et al., 2015). Accordingly, this isolate was
167 designated as *A. bereziniae*-like although whole-genome sequencing could aid to
168 clarify the taxonomic position of this isolate. Nevertheless, we acknowledge that
169 additional isolates with similar characteristics are needed before proposing the
170 existence of new taxa.
171 *3.2. Susceptibility to antimicrobial agents and epidemiological typing*
172 Antimicrobial susceptibility testing of the selected isolates by gradient diffusion and
173 broth microdilution (colistin) showed low MIC values to all the antimicrobial agents
174 tested except for chloramphenicol, to which MIC values below 8 mg/L were only
175 observed in both *A. pittii* isolates representative from fingerprint D (Table 1). Of note,
176 all isolates showed baseline MIC values between 3 and 6 μg/mL to ceftazidime and
177 between 1 and 4 μg/mL to cefepime, which might be attributed to the intrinsic
178 resistance of *Acinetobacter* spp. to cephalosporins. These results are in agreement
179 with those of Rafei et al. (2015), that showed susceptibility to most antimicrobials in
180 *Acinetobacter* spp. recovered from soil, animals and food products, with only a few

181 carbapenem-resistant isolates that were associated to the carriage of acquired OXA-
182 type carbapenemases, and also with those from Hamouda et al. (2011) that reported
183 high MIC values to chloramphenicol as well as similar baseline MIC values to
184 ceftazidime. In contrast, Carvalheira et al. (2017a) showed higher prevalence of
185 resistance to cephalosporins and quinolones in *Acinetobacter* spp. isolated from meat
186 (43.5% and 42.9%, respectively), and surprisingly high prevalence of resistance to
187 colistin and polymyxin B (41.7% and 35.1%, respectively), although MIC values
188 were not provided and a wider variety of *Acinetobacter* species were reported.
189 MLST studies identified the *A. baumannii* isolate as belonging to ST273, which had
190 been previously reported in 2000 from a hospitalised patient in Spain and had also
191 been identified from a calf in Switzerland in 2013 (Lupo et al., 2014). ST273 is
192 clustered into clonal complex (CC) CC33, which contains STs related to clinical
193 isolates. The *A. pittii* isolates from fingerprint D were assigned to ST312, an ST
194 previously described from a patient in Belgium in 2009 but to our knowledge, never
195 reported in animals and not included in any CC. The *A. dijkshoorniae* and *A.*
196 *bereziniae*-like isolates and the *A. pittii* isolate from fingerprint B presented novel
197 MLST alleles and were assigned new sequence types (ST1256, ST1258 and ST1257,
198 respectively) by the curators at https://pubmlst.org/. None of them clustered in any
199 CC either.
200 *3.3. Final remarks*
201 The identification of *Acinetobacter* isolates belonging to several species of the ACB
202 group in raw meat samples is of particular concern, since these species are usually
203 associated with the clinical setting. Previous studies had already reported the presence
204 of species belonging to this group from diverse meat samples, including poultry,
205 swine and beef, although members of the ACB group were not necessarily the most

206 prevalent species (Carvalheira et al., 2017a). In our study, *Acinetobacter* isolates were
207 only recovered from beef samples and the majority of isolates were identified as *A.*
208 *pittii*. Interestingly, the authors from a study in Lebanon (Rafei et al*.,* 2015) also
209 reported the presence of *A. baumannii, A. pittii* and *A. bereziniae* in cow meat
210 samples, with a prevalence similar to that observed in our investigation (28% and
211 27%, respectively) and *A. pittii* was again the predominant *Acinetobacter* species.
212 Multidrug resistant *Acinetobacter* spp. from the ACB complex currently represent a
213 serious threat to public health but, despite some of the *Acinetobacter* isolates
214 recovered in the present study showing genetic links to clinical isolates, all isolates
215 were susceptible to clinically relevant antibiotics and their overall prevalence was low
216 (8.6%). Nevertheless, contaminated meat should not be neglected as a source for the
217 transmission of *Acinetobacter* spp. into domestic and hospital settings where it may
218 also contribute to the evolution of clinical lineages which, ultimately, might
219 accumulate resistance genes.
220 It is also worth mentioning that the actual burden of *Acinetobacter* spp. from market
221 meat samples may be much higher than what was shown in our study, since specific
222 enrichment media or pre-enrichment steps for *Acinetobacter* were not used.
223 To our best knowledge, we also report here the first identification of the recently
224 described *A. dijkshoorniae* in meat samples of animal origin as well as its first
225 identification in Peru.
226

Availability of data and material

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

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334

335

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

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

342

							MIC (µg/mL)											
PFGE	**n**	**Calf**	**Area**	**Isolate**	**Species**	**ST**	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-7T	*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

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