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ORIGINAL RESEARCH PAPER

Clonal dissemination of *Acinetobacter radioresistens* among Humboldt penguins (*Spheniscus humboldti*) inhabiting a barren northern Peruvian island

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ABSTRACT

Acinetobacter spp. are often isolated from natural sources, but knowledge about their presence in wild animals is fragmented and uncomplete. The present study aimed to characterize a series of *Acinetobacter radioresistens* isolated from Humboldt penguins (*Spheniscus humboldti*). Fifteen Humboldt penguins from an inhabited northern Peruvian island were sampled. Microorganisms were identified by MALDI-TOF MS. Antibiotic susceptibility to 12 antimicrobial agents was established, and clonal relationships were determined. A representative isolate was selected for whole genome sequencing (WGS). *A. radioresistens* were isolated from the feces of 12 (80%) Humboldt penguins, being susceptible to all the antimicrobial agents tested, except eight cefotaxime-intermediate isolates. All *A. radioresistens* were clonally related. WGS showed that the isolate belonged to ST1972, the presence of two chromosomal encoded carbapenemases (bla_{OXA-23} and a putative subclass B3 metallo-β-lactamase), and a series of point mutations in antibiotic-resistance related chromosomal genes, which were considered as polymorphisms. In addition, a few virulence factors, including a capsule-encoding operon, superoxide dismutases, catalases, phospholipases and a siderophore receptor were identified. The present results suggest that *A. radioresistens* may be a common member of the gut microbiota of Humboldt penguins, but further studies in other geographical areas are needed to establish this finding.

KEYWORDS

Acinetobacter spp., one health, South America, antibiotic susceptibility, OXA-23

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INTRODUCTION

Several members of the genus *Acinetobacter* are often isolated as a cause of human diseases, with *Acinetobacter baumannii* being the most commonly identified in hospital settings [1, 2]. Nevertheless, most of the recognized *Acinetobacter* species are commonly recovered from other sources, including marketed food, pets, surfaces or wild animals [3–7].

Acinetobacter radioresistens was first identified in cotton and soil samples in 1988 [6]. *A. radioresistens* is a rare cause of human infections, with a role as opportunistic pathogen having been confirmed [8, 9]. Of note, *A. radioresistens* from human infections often present high antibiotic susceptibility levels [8–10], different to that reported for *A. baumannii* [1, 2, 11, 12]

It is remarkable that the OXA-23 group, a common cause of carbapenem resistance in *A. baumannii* [1, 13], is considered intrinsic of this microorganism [14]. Nevertheless, in *A. radioresistens*, this gene usually does not result in carbapenem resistance, and it has been proposed that in the absence of an upstream insertion sequence, such as ISA*cra1*, that may provide a strong promoter sequence, it is not expressed, or is expressed at low basal levels [14, 15].

Outside clinical settings, *A. radioresistens* has been isolated from other sources, such as inanimate surfaces, human skin or Antarctic soils [13, 16, 17]. Regarding animals, *A. radioresistens* has been recovered from different human-related animal species, such as cats or dogs, horses and chickens [4, 7, 18], as well as exotic pets, including chameleons, tortoises or geckos, among others [19], being scarcely reported in wild animals, such as Fitzinger's robber frog (*Craugastor fitzingeri*) or yellow-legged gulls (*Larus michahellis*) [18, 20].

In this scenario, the present study aimed to characterize a series of *A. radioresistens* recovered from Humboldt Penguins (*Spheniscus humboldti*) on an uninhabited guano inshore island in northern Peru.

MATERIAL AND METHODS

Sampling area

The sampling was conducted in November-December 2019 on Guañape Norte Island (8°32′41″S, 78°57′49″W). Guañape Norte is a barren rocky island spanning 0.34 km^2, situated 16 km off the Peruvian coasts within the *Reserva Nacional Sistema de Islas, Islotes y Puntas Guaneras* (a Peruvian Marine Protected Area). The island is uninhabited, except for two permanent rangers stationed there. However, it is visited by hundreds of guano harvesters at intervals of 4–5 years, with the last guano campaign dating back to 2014. Meanwhile, access to the island for researchers is restricted to occasional brief *ad-hoc* visits. Since 2019, small-scaled touristic boats have sporadically approached the island, but landing is prohibited (CZ, personal communication).

The island serves as a significant seabird breeding site, with an estimated population of 1802 Humboldt Penguins, coexisting with thousands of Guanays (*Leucocarbo bougainvilliorum*) and Peruvian Boobies (*Sula variegata*). Additionally, the presence of sea mammals, including the South American Sea Lion (*Otaria byronia*), is commonly observed on the island [21].

Humboldt penguins

Fifteen Humboldt Penguin chicks undergoing rearing were carefully captured for the study by gently extracting them from their nests using a noose attached to a 2-m pole. The noose was delicately secured around their necks and pulled out, allowing the penguins to be restrained by hand [22, 23]. The entire handling process took less than 1 min. Cloacal swabs were collected, transported to the laboratory (12–14 days) and cultured as previously described [3].

Culture and identification

Recovered microorganisms were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a MALDI Biotyper Sirius instrument (MALDI Biotyper®, Bruker Daltonics GmbH & Co. KG, Bremen, Germany) running in positive linear mode and using the MBT Compass v4.1 software package and the MBT Compass reference library (version 2022) for taxonomic identification. When more than one microorganism belonging to the same species was recovered from the same sample, only one was considered for further analysis.

Detection of *bla*$_{\text{OXA-23}}$

The presence of the *bla*$_{\text{OXA-23}}$ group gene (thereafter noted as *bla*$_{\text{OXA-23G}}$) was established by polymerase chain reaction following standard procedures [1]. The *bla*$_{\text{OXA-23G}}$-producing the *A. baumannii* 308 isolate was used as a positive control [24].

Antimicrobial resistance

Antimicrobial resistance levels to cefotaxime, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, tetracycline and tigecycline were established by disk diffusion in accordance with the Clinical & Laboratory Standards Institute (CLSI) guidelines [25]. Meanwhile, colistin resistance was established by agar-spot as previously described [26]. In the absence of CLSI/European Committee on Antimicrobial Susceptibility Testing/Food and Drug Administration criteria [25, 27, 28], the tigecycline susceptibility breakpoints considered were; susceptible ≥16 mm, intermediate 13–15 mm, resistant ≤12 mm as proposed by Jones et al. [29].

Clonal relationships

Clonal relationships were established by pulsed field gel electrophoresis (PFGE) and by Fourier-transform infrared spectroscopy (FTIR). PFGE was performed as described previously [30], using genomic digestions with the *Apa*I restriction enzyme and a CHEF-DRIII system (Bio-Rad

Laboratories, Hercules, CA, USA). Band patterns were compared with the InfoQuest TM FPv.5.4 software (Bio-Rad Laboratories, Hercules, CA, USA) and the unweighted pair group method with arithmetic mean was used for cluster analysis based on the Dice similarity coefficient. Using bandwidth tolerance and optimization values set at 1.5 and 1%, respectively, isolates were considered to belong to the same PFGE cluster (pulsetype) if their Dice similarity index was ≥85% [31].

For FTIR spectroscopy, infrared absorption spectra were captured using a Bruker IR Biotyper instrument (Bruker Daltonic, GmbH & Co. KG, Bremen, Germany) running on the IR Biotyper software version 3.1 and on default settings (32 scans per technical replicate; spectral resolution, 6 cm^{-1}; apodization function, Blackman-Harris 3-term; zero-filling factor, 4). Measurements were carefully checked and spectra that did not meet the quality criteria (0.4 < absorption <2; signal-to-noise ratio, >40; fringes [$\times 10^{-6}$], <100) were removed from the analysis. Spectra were acquired within the 4,000–400 cm^{-1} wavenumber range but spectra processing for initial clustering of the isolates were based on the second derivative of the 1,300–800 cm^{-1} region that is mostly associated with absorption data from cell wall carbohydrates. Cluster analysis was performed using Euclidean distances and the UPGMA linkage algorithm.

Both PFGE and FTIR cluster analysis included three clonally-related OXA-23-producing *A. baumannii* isolates for outgroup comparison [12].

DNA extraction and sequencing

DNA was extracted using the ZymoBIOMICS™ DNA miniprep Kit (Zymo Research, Irvine, USA), according to the manufacturer's protocol. DNA quality and quantity were determined using the NanoDrop™ 2000 Spectrophotometer and the Quantus™ Fluorometer and QuantiFluor® dsDNA System (Promega, Madison, USA), respectively.

Illumina libraries were prepared with the Illumina DNA prep kit (Illumina, San Diego, USA) and Nextera DNA CD Indexes (Illumina, San Diego, USA), using approximately 400 ng of input DNA. The libraries were normalized using a bead-based procedure and pooled together at equal volume. The pooled library was denatured and sequenced using Miseq reagent version 2 (Illumina, Inc. San Diego, CA) on an Illumina Miseq platform with 2 × 150 paired-end chemistry.

Genome assembly and quality assessment

Illumina fastQ reads assembly was conducted using Bactopia v2.2.0 [32]. Shovill v1.1.0 (https://github.com/tseemann/shovill) was used to perform the genome assembly, whereas quast v5.0.2 [33] was used to evaluate the genomic assembly. The resulting genome comprised 27 contigs with an overall coverage of 70×.

Bioinformatic analysis

Detection of transferable antimicrobial and heavy metal resistance genes. AMRFinderPlus v3.11.20 was used for detection of antimicrobial and antimicrobial resistance genes. This software uses the curated Reference Gene Database of the National Center for Biotechnology Information.

Detection of chromosomal point mutations. Mutations in chromosomal genes involved in the development of antimicrobial resistance in *gyrA*, *gyrB*, *parC*, *parE* (quinolones), *rpoB* (rifampicins), *rplD*, *rplV* (macrolides), *rpsE*, *rpsG*, (aminoglycosides), *mrcA*, *mrcB*, *mrdA*, *ftsI* (β-lactams), *lpxA*, *lpxB*, *lpxC*, *lpxD*, *lpxL*, *lpxM*, *lpsB* and *pmrC* genes (polymyxins) as well as in 16s rRNA (resistance to tetracyclines) and 23s rRNA (resistance to macrolides and anfenicols) were analyzed comparing the obtained sequences with both the *A. radioresistens* GenBank reference genome LH6 (Accession number CP030031.1) and the type strain DSM 6976 (Accession number AP019740.1). Only amino acid differences (or base differences for 16s rRNA and 23s rRNA) shared with both reference genomes were considered.

Detection of virulence factors. The automatic pipeline VF analyzer (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) was employed for analysis of virulence factor-encoding genes, using raw FASTA sequence as input.

Phylogenomic comparison and pangenome. A total of 153 *A. radioresistens* genomes and corresponding metadata were downloaded using the NCBI datasets command-line tools on January 10, 2024, considering both draft genomes and complete assemblies. Genome metrics were obtained with Quast v. 5.2.0 [34], discarding 53 genomes due to quality issues. To ensure reducing bias from different annotation algorithms, the 100 remaining genomes were annotated using Prokka v. 1.14.6. [35]. The pangenome was calculated using Roary v. 3.13 and Snp-sites v. 2.5.1 was employed to extract SNPs from the multi-alignment [36, 37]. The phylogenomic tree was then constructed using IQ-TREE v. 2.06. with automatic model selection and ultrafast Bootstrap for 1,000 replicates [38–40]. Tree visualization and pangenome representation were obtained with FigTree v. 1.4.4. and Phandango respectively [41, 42]. The definition criteria for Core and Soft genome was the presence in the 99% and 95% of analyzed genomes.

Afterwards, the closest genome and the closest confirmed human pathogenic clinical isolates were selected to calculate the average nucleotide identity (ANI), using the OrthoANIu algorithm [43].

Multilocus sequence typing detection

In the absence of a specific sequence typing (ST) scheme for *A. radioresistens*, the ST pattern was established following the *A. baumannii* Pasteur scheme (https://pubmlst.org/organisms/acinetobacter-baumannii), which also considers *Acinetobacter* species other than *A. baumannii*.

Lipooligosaccharide outer core and capsule. The presence and type of genes involved in lipooligosaccharide outer core (OCL) and capsule synthesis was determined using Kaptive (https://kaptive-web.erc.monash.edu/).

Data availability statement

Genomic data are recorded under Genome assembly and Sequencing project ASM3404387v1 and JAXCJJ000000000.1 respectively, with the accession code: GCA_034043875.1; Sequence Read Archive accession for the fastQ reads is SRR26939120. Access to the Illumina fastQ reads and the assembled genome is available through the NCBI BioProject PRJNA1044581.

Ethics approval

This study was approved by the Institutional Ethics Committee in Research with Animals and Biodiversity (Code N° 076-CIEI-AB-CIENTÍFICA-2020) of the Universidad Científica del Sur. Permits to work on Guañape Norte island and swabbing the birds were issued by SERNANP (RJ-012-2019-SERNANP-RNSIIPG). Likewise, permits to use guano island installations were issued by Agrorural (Oficio 0249-2019-MINAGRI-DVDIARAGRORURAL-DAB).

RESULTS

In the course of the study, 23 non-duplicate microorganisms were recovered from 15 Humboldt penguins sampled, including *A. radioresistens* (12 isolates), *K. oxytoca* (4 isolates), *Pseudomonas* spp. (2 isolates), *Pseudomonas putida* (2 isolates), *Pseudomomas aeruginosa* (1 isolate), *Morganella morganii* (1 isolate) and *Pantoea* spp. (1 isolate). In all cases the identification at the species level was established by MALDI-TOF MS.

The 12 non-duplicate *A. radioresistens* were included in further studies. The *A. radioresistens* isolates presented the $bla_{\text{OXA-23}}$ gene and showed high levels of antibiotic susceptibility, with only 8 isolates showing intermediate resistance to cefotaxime. Interestingly, the clonal analysis, performed by either PFGE or FTIR spectroscopy, showed that all the isolates were genetically related and could be included within the same clonal cluster (Fig. 1). Subsequently, a representative isolate, designated as *A. radioresistens* 4A, was further selected for a full characterization through whole genome sequencing (WGS) analysis (Fig. 2).

WGS confirmed the presence of $bla_{\text{OXA-23G}}$, which was identified as the $bla_{\text{OXA-23}}$ gene. Additionally, despite the lack of resistance to carbapenems, the presence of a chromosomal putative subclass B3 metallo-beta-lactamase showing 100% identity with that of the *A. radioresistens* strain FDAARGOS_731 (GenBank access: CP059684.1), was also detected. Alterations in chromosomal genes either directly encoding antibiotic targets or involved in metabolic processes that may lead to antibiotic-susceptible or antibiotic-resistant phenotypes were sought, but while a series of point mutations were detected, no apparent effect on antibiotic resistance was found, and they were thereby classified as polymorphism (Table 1). Meanwhile, no gene involved in resistance to heavy metals was detected. On the other side, the analysis also showed the presence of several virulence factors, including genes related to OCL, high-affinity iron-chelating compound receptors, phospholipases, among others (Table 2), and allowed assigning the representative isolate to ST1972. Regarding OCL, the analysis detected eight genes close related to OCL3 (identities varying from 94.3% to 100%), with an overall identity degree of 91.4%. Of note, the homologous gene to gtr_{OC1} was not identified (Table 2). Regarding capsule, the analysis only detected the presence of 8 out of 20 genes involved in its synthesis, and thereby was not further examined.

The obtained phylogenomic tree showed that, in general, *A. radioresistens* can be divided in two major clades, and that confirmed human pathogenic *A. radioresistens* are not restricted to one clade only. The genome from this study was more related to GCA_023130635.1 (ANI 99.96%), which is a

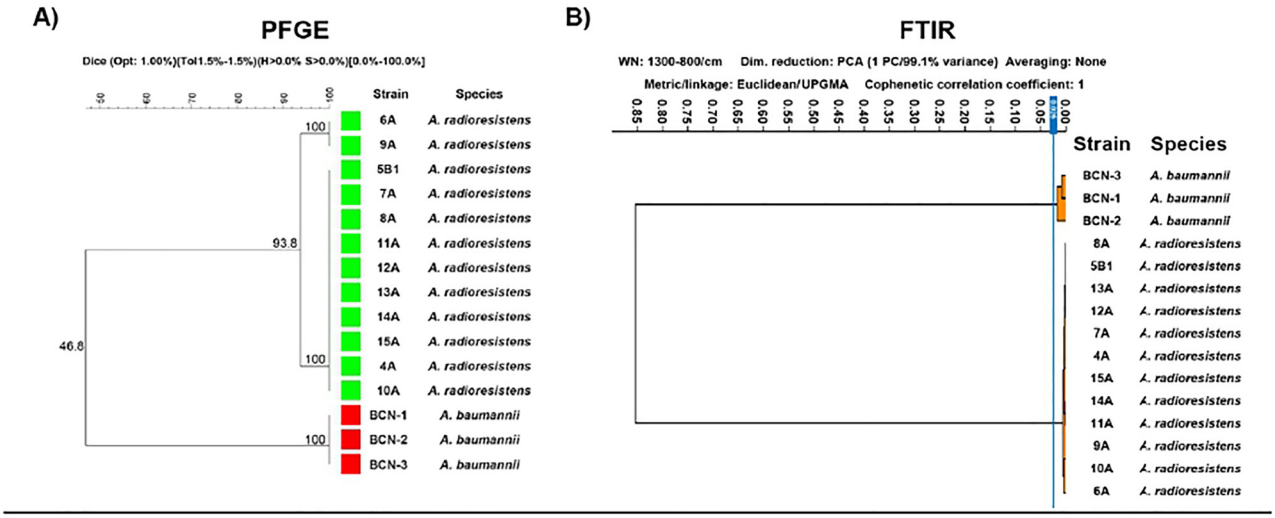

Fig. 1. **(A)** PFGE and **(B)** FTIR dendrograms depicting the clonal relatedness of *Acinetobacter radioresistens* isolates recovered from Humboldt penguins and three *Acinetobacter baumannii* isolates included as an outgroup

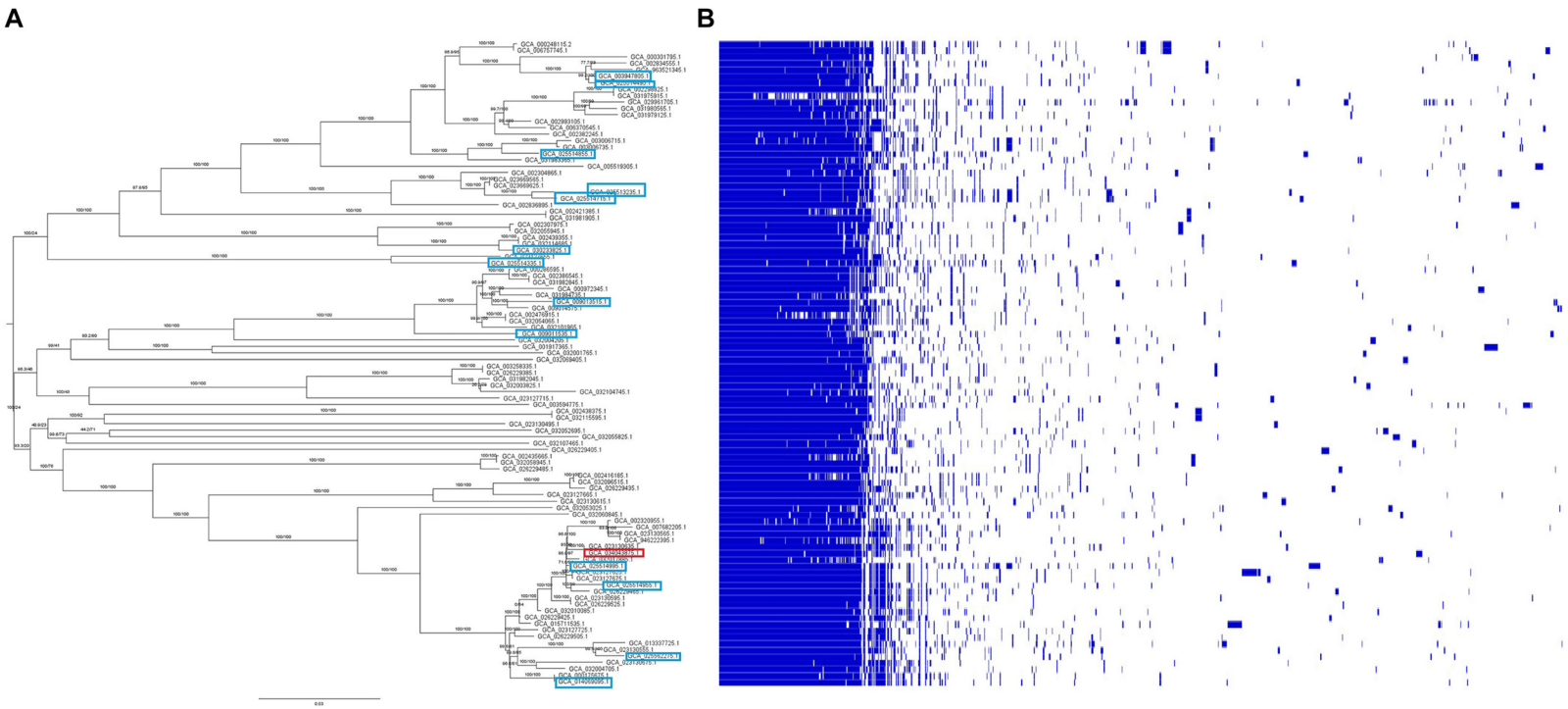

Fig. 2. Phylogenomic tree and pangenome representation of the analyzed genomes

A: Phylogenomic tree.

The genome from this study is shown within the red box, whilst Genomes in light blue boxes are confirmed clinical isolates/pathogenic *A. radioresistens*.

B: Pangenome of *Acinetobacter radioresistens*.

The core genome is considered when the genes are present in 99% of the isolates.

Table 1. Chromosomal point mutations in antibiotic resistance-related genes

Genes	Point Mutations[1]	Resistance[2]
gyrA	A510V, V529I, T684M, E981Q	Quinolones
gyrB	R219C	Quinolones
parC	E356G, H395R	Quinolones
16S rRNA	T1449C (DNA)	Tetracyclines
mrcB (PBP1b)	D85N, S134A, E336D	β-lactams
mrdA (PBP2)	A402T, T660M	β-lactams
ftsI (PBP3)	P15S, N156K	β-lactams
lpxA	I31V	Polymyxins
lpxB	P161S, P343A	Polymyxins
lpxC	K8R	Polymyxins
lpsB	S71P, K74R, I95T, A125E, Q370R	Polymyxins
pmrB	A55T, R260K	Polymyxins

No alterations were found in the remaining genes analyzed.
[1] Only reported differences common to both strain LH6 (GenBank: CP030031.1) and strain DSM 6976 (GenBank: AP019740.1).
[2] Antibiotic resistance which has been related to the presence of specific alterations in these genes.

Table 2. Virulence factors

Genes	Virulence factor
orfs 2549-2555; orfs 2561-2569	Lipooligosaccharide outer core locus
plcN	Phospholipase C
plD-1	Phospholipase D
plD-2	Phospholipase D
fepA	Siderophore receptor
bfr-1	bacterioferritin
bfr-2	bacterioferritin
katA	Catalase
katB	Catalase
katD	Catalase
sodB	Superoxide dismutase
sodC	Superoxide dismutase

Open reading frames (Orfs) indicated following numeration of the draft genome of isolate 4A (GenBank access JAXCJJ000000000.1).

German isolate from a human urine sample collected in 2013. Moreover, the other two closest pathogenic isolates GCA_025514995.1 and GCA_025514955.1 (ANI 99.82% and 99.81% respectively) were collected in 2019 from a Hospital in the United States of America. The pangenome showed a total of 14,209 genes, with only "core" and "soft core" genes being 1,108 and 901 respectively. Many of the accessory genes classified as hypothetical proteins, plasmid genes, insertion sequences and transposases.

DISCUSSION

A. radioresistens is an environmental microorganism [6], which has also been involved in opportunistic infections [8, 9, 17]. While the isolation of *A. radioresistens* in wild animals has previously been described, with descriptions in frogs or yellow-legged gulls [18, 20], in the present study the common presence of *A. radioresistens* as an apparent commensal inhabitant of the gut microbiota of Humboldt penguins is reported for the first time. Of note, neither selective media for detection of *Acinetobacter* spp., nor culture-independent techniques for the detection of *Acinetobacter* species were used. Nevertheless, while most studies focused on the direct or indirect detection of microorganisms in wild or captive Humboldt penguins have been addressed to pathogenic issues [44–46], in one study describing the gut microbiota of captive Humboldt penguins, the presence of *Acinetobacter* spp. ranked among the taxa with the highest relative abundance [47].

In agreement with a low anthropic pressure and feeding habits, the present isolates were susceptible to all the antimicrobial agents tested, except for 8 cefotaxime intermediate isolates. This finding corroborates similar results regarding the recovery of *Acinetobacter* spp. and *Pseudomonas* spp. from cloacal swabs from Peruvian boobies inhabiting the same island [3]. Of note, *Escherichia coli* recovered from the above-mentioned Peruvian boobies presented resistance to several antimicrobial agents [3]. It is worth mentioning that clinical reports of *A. radioresistens* often shows a similar scenario of antibiotic susceptibility [8–10]. Eight isolates displayed intermediate susceptibility to cefotaxime, with no specific mechanisms being identified. Thereby this phenomenon might be related to differences in the expression levels of chromosomal efflux pumps, with this role confirmed in other species of the genera [48].

According to the results of two different techniques, all the isolates belonged to the same clonal cluster, likely related to the density of the Humboldt penguin colony which might favor microorganism sharing. This finding showing than in densely populated bird colonies, microorganisms either infecting birds or inhabiting their microbiotas may easily be dispersed and shared. Of note, an unidentified fully antibiotic-susceptible *Acinetobacter* spp. was recovered from a Peruvian booby during the same sampling [3].

Despite the above-mentioned antibiotic susceptibility, the isolates presented two carbapenemases and several antibiotic-target alterations. OXA-23 is intrinsic of this microorganism, but its standard low level of expression does not allow the development of resistance to carbapenems [11]. Nevertheless, the mobilization of OXA-23, due to insertion sequences, to other members of the genus (such as *A. baumannii*) results in high levels of resistance to carbapenems due to increasing levels of expression [11]. On the other hand, there is limited data regarding the putative subclass B3 metallo-beta-lactamase that also appears to be intrinsic of *A. radioresistens* [49], but a similar scenario of low/null expression in the absence of deregulation seems plausible. Of note, while a series of point mutations in several genes commonly known to be associated with the development of antibiotic resistance were identified, the overall antibiotic susceptibility levels strongly suggest the presence of nucleotide polymorphisms with no impact on

the development of antibiotic resistance. Finally, no specific reason was found to explain the isolates intermediate to cefotaxime.

The presence of virulence factors, including siderophore acquisition system, shows the potential of these isolates to produce infections in both humans and animals. Despite 153 *A. radioresistens* genomes are present in GenBank, no specific data about the OCL diversity in this specie has been found. OCL3 it has been described as present in international clones (IC) of high risk of *A. baumannii* such as IC1 or IC2 [50]. Of note, potential health issues related to the presence of *A. radioresistens* were not observed in the penguins sampled or in posterior reports from the Humboldt penguin colony. These findings suggest that *A. radioresistens* is a commensal, or at least innocuous resident, in the gut microbiota of Humboldt penguins.

A search in https://pubmlst.org/organisms/acinetobacter-baumannii showed that ST1972 has only been reported twice: isolates FDAArgos_731 (GenBank access: CP059684.1) and MGYG-HGUT-01344 (GenBank access: CABKOV010000001-CABKOV010000018). In both cases *A. radioresistens* were from a human origin (skin and gut). This finding suggests ease of interspecies spread from human to animals or vice versa.

While there are previous genomic comparison studies of *Acinetobacter* spp. which naturally include *A. radioresistens* [51], to the best of our knowledge, this study includes the first pangenomic comparison amongst genomes of *A. radioresistens* only. Of note, confirmed human pathogenic isolates were non-grouped, thereby no specific association of them with present isolate was done. Regarding isolates belonging to ST1972 both were excluded for comparisons due to genome quality issues.

The main limitation of this study is the time lapse from samples acquisition to laboratory culture, which was related to the nature of the sampling. In addition, the same delay in sample processing might affect bacterial viability of several microorganisms, resulting facilitating the isolation of *A. radioresistens*.

In summary, the present study highlights the clonal spread of *A. radioresistens* in a Humboldt penguin colony. Further studies in other geographical areas are needed to establish the commonness of this microorganism as an inhabitant of the Humboldt penguin gut, to determine if it belongs to the normal gut microbiota or if the present results show a local phenomenon.

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