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A high-risk carbapenem-resistant *Pseudomonas aeruginosa* clone detected in red deer (*Cervus elaphus*) from Portugal

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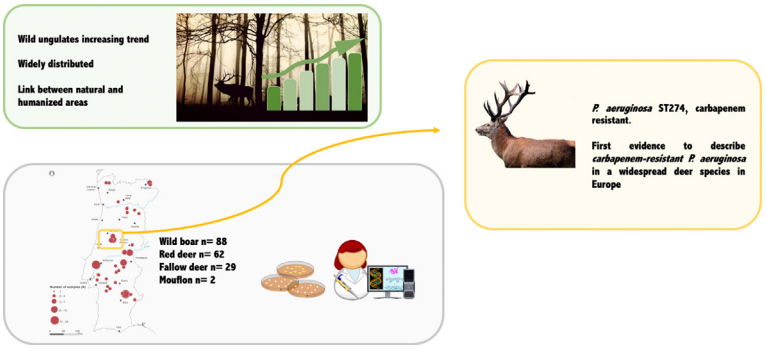
HIGHLIGHTS

- Occurrence of carbapenem-resistant bacteria in wild ungulates has been investigated.
- First report of carbapenem-resistant *Pseudomonas aeruginosa* in deer in Europe
- Description of a high-risk close (ST274), usually associated to healthcare settings
- Red deer may contribute to the spread of AMR determinants.

GRAPHICAL ABSTRACT



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ABSTRACT

Pseudomonas aeruginosa is a ubiquitous bacterium, successfully exploiting a variety of environmental niches due to its remarkable metabolic versatility. The World Health Organization classifies *P. aeruginosa* as a "priority pathogen" due to its a great ability to overcome the action of antimicrobials, including carbapenems. Hitherto, most studies have focused on clinical settings from humans, but much less on animal and environmental settings, particularly on wildlife. In this work, we report the isolation of a carbapenem-resistant *Pseudomonas aeruginosa* strain recovered from the faeces of a red deer adult female sampled in a humanized area. This isolate was obtained during a nationwide survey on antimicrobial resistance in wildlife aimed to determine the occurrence of carbapenem-resistant bacteria among 181 widely distributed wild ungulates. This *P. aeruginosa* isolate was found to be a high-risk clone, belonging to the sequence type (ST) 274. The genomic analysis of *P. aeruginosa* isolate UP4, classified this isolate as belonging to serogroup O3, which was also found to harbour the genes bla_{PAO}, bla_{PDC-24}, $bla_{OXA-486}$ (encoding resistance to beta-lactams), *aph(3′)-IIb* (aminoglycosides resistance), *fosA* (fosfomycin resistance) and *catB7* (chloramphenicol resistance). Antimicrobial susceptibility screening, according to EUCAST, showed resistance to imipenem and intermediate resistance to meropenem and doripenem. To our knowledge, this is the first description of carbapenem-resistant *P. aeruginosa* in deer in Europe. Our results highlight the importance of wild ungulates either as victims of human activity or amplifiers of AMR, either way with potential impacts on animal, human and ecosystem health, since excretion of AMR bacteria might directly or indirectly contaminate other animals and the surrounding environment, perpetuating the spill-over and chain dissemination of AMR determinants.

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1. Introduction

The gram-negative *Pseudomonas* genus contains over 60 species with high metabolic versatility and ecological diversity, which are grounded on their bacterial genomes that include several genetic mobile elements, as well as intrinsic and acquired resistance mechanisms (Lister et al., 2009). This genus adaptation plasticity to different ecological conditions enables the colonization of a wide variety of environmental matrices, from terrestrial to aquatic niches (Moradali et al., 2017; Nadimpalli et al., 2020). From the wide variety of species that this genus encompasses, the opportunistic pathogen *Pseudomonas aeruginosa* is undoubtedly of medical and veterinary relevance (Moradali et al., 2017). This pathogen causes nosocomial infections, pulmonary infections in cystic fibrosis patients, disseminated infections in humans with compromised immune systems (Moradali et al., 2017), in livestock (e.g. causing mastitis in dairy cows) and companion animals (e.g. urinary infections in dogs González-Torralba et al., 2016) but it is usually present in the normal gut microbiota in healthy humans or animals (Lister et al., 2009). According to the World Health Organization, *P. aeruginosa* is one of the most critical antibiotic resistant (AMR) "priority pathogens", as this bacterium shows a great ability to bypass the action of antibiotics (World Health Organization, 2017). Due to its ubiquity, ability to successfully exploit a variety of environmental niches and acquired AMR arising from selective pressure imposed by sub-inhibitory concentrations of antibiotics, *P. aeruginosa* circulating strains are now resistant to several antibiotic classes, including carbapenems (Ciofu and Tolker-Nielsen, 2019). Carbapenems are last-resort antibiotics that are used for the treatment of serious infections by multidrug-resistant (MDR) bacteria, including *P. aeruginosa*. Carbapenem-resistant *Pseudomonas aeruginosa* are one of the leading causes of health care-associated infections, with treatment being particularly difficult (Walters et al., 2019). Besides human settings reports of carbapenem-resistant *P. aeruginosa* in animals have also been published (Cabassi et al., 2017; Haenni et al., 2017), highlighting its global widespread.

Environmental reservoirs of antimicrobial resistant bacteria (AMR) have been increasingly hypothesized and discussed as potential sources for human infection (Mills and Lee, 2019). Even though it is known that *P. aeruginosa* prosper in different niches, hitherto most of the studies have focused on clinical settings and much less on animal and environmental settings (Rodrigues et al., 2021). Surveillance studies of *Pseudomonas* spp. in wildlife, particularly carbapenem-resistant *P. aeruginosa*, are an underexplored area that may pose risks to humans, animals, and the surrounding environment (Hernando-Amado et al., 2019).

In this sense, wild ungulates have been suggested as sentinel or bioindicator species to trace the spread of AMR across ecological gradients (Torres et al., 2020, 2021) due to their ubiquity (e.g., wild ungulates have wide distribution areas and have expanded in number and distribution all over Europe in the last decades), and relationship between natural and anthropogenic areas. As the role of wildlife in the emergence and dissemination of AMR *P. aeruginosa* in wildlife is understudied, the main aims of this study were to determine the occurrence of carbapenem-resistant bacterial isolates among faecal samples of widely distributed wild ungulates on a national scale, and to analyze their phenotypic and genotypic characteristics, including AMR profiles and molecular types.

2. Materials and methods

2.1. Sampling and bacterial isolation

Sampling of wild ungulates was completed during two hunting seasons (October to February; 2018/2019 and 2019/2020), from 36 different hunting grounds, located in 15 of the 18 districts of continental Portugal (Fig. 1).

A total of 181 faecal samples were opportunistically collected from legally hunted animals ($n = 88$ from wild boar, *Sus scrofa*; $n = 62$ from red deer, *Cervus elaphus*; $n = 29$ from fallow deer, *Dama dama* and $n = 2$ from mouflon, *Ovis orientalis musimon*) within 1–3 h after death. No animals were sacrificed for the purposes of this study. None of the authors was responsible for the death of any animals. Wild ungulates were hunted by professional hunters during legal game activities. No animal were hunted for the project sake, but on the annually hunting activities following the Portuguese Hunting Law (http://www.icnf.pt/portal/caca/legis/reg-lei-bas-ger-caca); all applicable institutional and/or national/international guidelines for the care and use of animals have been followed.

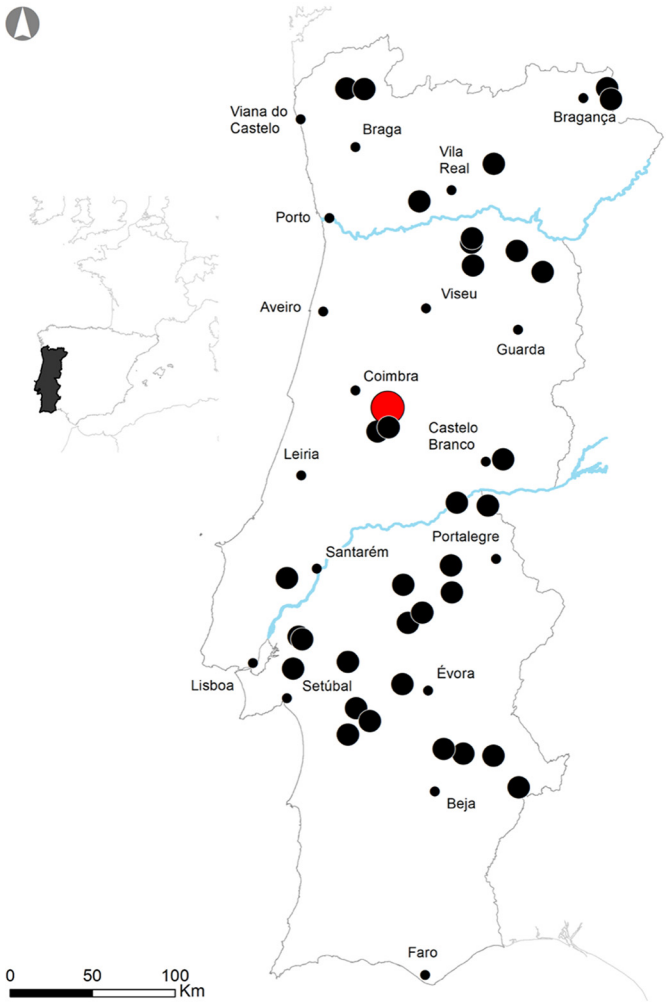

Fig. 1. Locations of sample collection (red circle indicates the location of the carbapenem-resistant isolate).

The faecal samples were collected directly from the rectus of the animals while the carcasses were being assessed by official veterinary inspectors, with sterilized material and subjected to refrigeration conditions during transportation to the laboratory, where they were stored at −20 °C until microbiological analysis shortly after. Faecal samples were thawed, submitted to enrichment with Tryptic Soy Broth (TSB, Liofilchem – Italy) and incubated without agitation at 37 °C for 24 h. To select carbapenem-resistant isolates, 100 μL of bacterial suspensions grown in the enrichment broth were inoculated onto the surface of MacConkey agar (Liofilchem – Italy) supplemented with meropenem (MRP, 0,5 μg/mL, Sigma-Aldrich - Germany), followed by incubation at 37 °C during 24 h (Palmeira et al., 2021). MacConkey agar (Liofilchem – Italy) without antibiotic was used as a growth positive control. *Escherichia coli* ATCC 25922 was used as quality control of selective and non-selective media. One colony per morphotype was streaked onto the antibiotic-selection plate, at 37 °C/24 h, for isolation and resistance confirmation purposes, as described previously (Palmeira et al., 2020).

2.2. Bacterial identification and antimicrobial susceptibility-testing

The disk-diffusion method was performed to evaluate the antimicrobial resistance profiles of isolated bacteria. The methodology was carried out according to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2021), using antibiotic disks (Oxoid – United Kingdom) for the following antibiotics: piperacillin (PRL, 30 μg), piperacillin plus tazobactam (TZP, 36 μg), ticarcillin (TIC, 75 μg), ticarcillin plus clavulanic acid (TCC, 85 μg), ceftazidime (CAZ, 10 μg), cefepime (FEP, 30 μg), aztreonam (ATM, 30 μg), meropenem (MRP,10 μg), imipenem (IMI, 10 μg), doripenem (DOR, 10 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), amikacin (AMK, 30 μg) and tobramycin (TOB, 10 μg). The Clinical and Laboratory Standard Institute (CLSI, 2020) standards were used for gentamycin (GEN 10 μg), netilmicin (NET, 30 μg) and norfloxacin (NOR, 10 μg). Presumptive identification was initially performed with CHROMagar Orientation (CHROMagar – France), followed by ID32GN galleries (Biomerieux – France) and confirmed by genome sequence analyses in the SpeciesFinder-2.0 Server (https://cge.cbs.dtu.dk/services/SpeciesFinder/) as described below. For meropenem, imipenem and colistin, minimal inhibitory concentration (MIC) was determined by the *E*-test (Liofilchem – Italy) and interpreted in accordance with EUCAST standards for clinical breakpoints and ECOFFs analysis (EUCAST, 2021).

2.3. Carbapenemases genes screening

Bacterial DNA from the carbapenem-resistant isolate was tested by PCR to screen carbapenemases genes. Three multiplex PCRs were performed, which include the most prevalent acquired carbapenemases genes (Table 1). The PCR reaction was performed under the followed conditions: 10 m at 94 °C, followed of 36 cycles of 30s at 94 °C, 40s at 52 °C, and 50s at 72 °C, and a final extension of 5 min at 72 °C (Poirel et al., 2011) and using Super Hot Master Mix (Bioron – Germany). Bacterial DNA from the carbapenem-resistant isolate was tested by PCR to screen carbapenemases genes. Three multiplex PCRs (Poirel et al., 2011) were performed, which include the most prevalent acquired carbapenemases genes, according table X, and using Super Hot Master Mix (Bioron – Germany).

2.4. Whole genome sequencing

Genomic DNA extraction, library preparation, sequencing, read trimming and assembly was performed according to the protocol of MicrobesNG (https://microbesng.uk/). Bacterial cells from a 50 μL cell suspension were lysed with 120 μL of TE buffer containing lysozyme and RNase A (ITW Reagents - Spain), followed by incubation at 37 °C for 25 min. SDS (Sigma-Aldrich - USA) and Proteinase K (VWR Chemicals - USA) was added and incubation at 65 °C for 5 min followed. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer (Qiagen - Germany). Quant-iT dsDNA HS (ThermoFisher Scientific - UK) assay was used to quantify DNA in an Eppendorf AF2200 plate reader (Eppendorf UK Ltd. - UK). Using the Nextera XT Library Prep Kit (Illumina - USA), the genomic DNA libraries were prepared following the manufacturer's protocol, and using a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG - Switzerland). Libraries were sequenced by Illumina using a 250 bp double paired end protocol. De novo assembly was performed using SPAdes version 3.7, and contigs were annotated using Prokka 1.11. AMR genes and typing analysis was carried out through the online tools available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org) and The Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/).

3. Results

A single carbapenem-resistant isolate (isolate UP4) was detected among the bacteria isolated from the 181 wild ungulates faecal samples, being this isolate phenotypically and molecularly identified as *Pseudomonas aeruginosa*. This isolate was obtained from a red deer adult female sampled in October of 2019 from central Portugal (Vilarinho, Coimbra -8,19374; 40,11618). The Multilocus Sequence Typing of *P. aeruginosa* isolate UP4 was determined by MLST 2.0 (version 2.0.4) tool (https://cge.cbs.dtu.dk/services/MLST/) according to the Warwick scheme and showing that this carbapenem-resistant isolate belongs to the sequence type (ST) 274, a high-risk clone, presenting the following housekeeping genes allelic profile *acsA23*, *aroE5*, *guaA11*, *mutL7*, *nouD1*, *ppsA12* and *trpE7*. Under the International Antigenic Typing Scheme (IATS), the genomic analysis by PAst 1.0 (https://cge.cbs.dtu.dk/services/PAst/) of the UP4 *P. aeruginosa* classified this isolate into serogroup O3.

Regarding antimicrobial susceptibility, the recovered isolate was susceptible to piperacillin, piperacillin plus tazobactam, ticarcillin, ticarcillin plus clavulanic acid, ceftazidime, cefepime, aztreonam, ciprofloxacin, levofloxacin, norfloxacin, gentamycin, netilmicin, amikacin, tobramycin and colistin. But showed resistance to imipenem and intermediate resistance profile to meropenem and doripenem, thereby corresponding to a non-wildtype phenotype according to the ECOFF values analysis. Minimal inhibitory concentration to imipenem and meropenem were 12 μg/mL and 6 μg/mL, respectively. None of the acquired carbapenemases genes

Table 1
Carbapenemases genes screening.

Multiplex	Gene	Primer	Sequence	Product size	PCR conditions
1	*bla*$_{IMP}$	IMP-F	5′-GGAATAGAGTGGCTTAAYTCTC-3′	232	10 m at 94 °C, followed of 36 cycles of 30 s at 94 °C, 40 s at 52 °C, and 50 s at 72 °C, and a final extension of 5 min at 72 °C
		IMP-R	5′-GGTTTAAYAAAACAACCACC-3′		
	bla$_{VIM}$	VIM-F	5′-GATGGTGTTTGGTCGCATA-3′	390	
		VIM-R	5′-CGAATGCGCAGCACCAG-3′		
	bla$_{SPM}$	SPM-F	5′-AAAATCTGGGTACGCAAACG-3′	271	
		SPM-R	5′-ACATTATCCGCTGGAACAGG-3′		
2	bla$_{KPC}$	KPC-F	5′-CGTCTAGTTCTGCTGTCTTG-3′	798	
		KPC-R	5′-CTTGTCATCCTTGTTAGGCG-3′		
	bla$_{NDM}$	NDM-F	5′-GGTTTGGCGATCTGGTTTTC-3′	621	
		NDM-R	5′-CGGAATGGCTCATCACGATC-3′		
	bla$_{OXA-48}$	OXA-F	5′-GCGTGGTTAAGGATGAACAC-3′	438	
		OXA-R	5′-CATCAAGTTCAACCCAACCG-3′		
	bla$_{BIC}$	BIC-F	5′-TATGCAGCTCCTTTAAGGGC-3′	537	
		BIC-R	5′-TCATTGGCGGTGCCGTACAC-3′		
	bla$_{AIM}$	AIM-F	5′-CTGAAGGTGTACGGAAACAC-3′	322	
		AIM-R	5′-GTTCGGCCACCTCGAATTG-3′		
	bla$_{GIM}$	GIM-F	5′-TCGACACACCTTGGTCTGAA-3′	477	
		GIM-R	5′-AACTTCCAACTTTGCCATGC-3′		
	bla$_{DIM}$	DIM-F	5′-GCTTGTCTTCGCTTGCTAACG-3′	699	
		DIM-R	5′-CGTTCGGCTGGATTGATTTG-3′		
	bla$_{SIM}$	SIM-F	5′-TACAAGGGATTCGGCATCG-3′	570	
		SIM-R	5′-TAATGGCCTGTTCCCATGTG-3′		

screened was detected by PCR. Nevertheless, analysis of the UP4 isolate's whole genome sequence by ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and RGI 5.2.0 (https://card.mcmaster.ca/analyze/rgi) evidenced that it harboured the genes bla_{PAO}, bla_{PDC-24}, $bla_{OXA-486}$ (resistance to beta-lactams), *aph(3′)-IIb* (resistance to aminoglycosides), *fosA* (resistance to fosfomycin) and *catB7* (resistance to chloramphenicol) (Table 2).

4. Discussion

In our study, 181 faecal samples of wild ungulates were collected from various locations across continental Portugal. Even though we only reported one carbapenem-resistant *P. aeruginosa* isolate, this is extremely significant. First, because carbapenems are critically important antibiotics restricted to human use and our findings show that carbapenem resistance may emerge in wild animals microbiota, specifically in this case in *P. aeruginosa*. Second, because it stresses that this resistance mechanism may not yet be widespread in wild ungulates in Portugal, as it is limited to only one animal and one location, meaning that we have identified a possible high-risk population and area, which surely deserves the design and implementation of proactive and targeted management programs. Additionally, more follow-up studies of this population are needed to identify potential risk factors. The sampling locations of this study were varied, reflecting a natural, semi-natural and a humanized gradient. The sample location where the carbapenem-resistant *P. aeruginosa* was collected is included in the humanized area, where human activity and density is high. Last-line antibiotics such as carbapenems (e.g., imipenem, meropenem which were identified in this study) are not expected to be used in wild animals. However it is widely acknowledge that the increasing usage of carbapenems in hospitals employs a selective pressure, which can ultimately foster the emergence of resistant *Pseudomonas* spp. clones in both clinical and community settings (Sievert et al., 2013). In fact, the intestinal tract is considered the most important reservoir of *P. aeruginosa* in clinical settings with gut colonization by this bacterium underlying the development of invasive infections. Therefore, due to our findings, we hypothesize that the deer populations where this isolate was reported are in close contact with humans or are victims of human-related activities that expose them close to selective pressure for the emergence of resistance. In agreement with this speculation, previous studies have also reported *Pseudomonas* spp. in wild birds near urban areas (Rodrigues et al., 2021).

All beta-lactamases detected (bla_{PAO}, bla_{PDC-24}, $bla_{OXA-486}$) in the genome of the UP4 isolate are considered native of *P. aeruginosa* and no acquired carbapenemases were found by PCR screening or WGS. However, UP4 shows reduced susceptibility to carbapenem antibiotics, but not to other beta-lactams, suggesting that their carbapenem-resistance occurs by a combination of resistance mechanisms, possibly other beta-lactamases, efflux pump overexpression or decreased cell permeability due to porin mutations/loss (European Committee on Antimicrobial Susceptibility Testing, 2017; Tsai et al., 2014). Conversely, the resistance genes to non-beta-lactam antibiotics detected *aph(3′)-IIb*, *fosA* and *catB7* confer resistance to aminoglycosides, fosfomycin and chloramphenicol, respectively. All these resistance genes have been well described in *P. aeruginosa* worldwide and frequently with chromosomal location (Kocsis et al., 2021). Our isolate is a high-risk clone of *P. aeruginosa* what by itself is of high significance, since it origin is a wild ungulate and this kind of clone is mostly associated with hospital settings, but their relevance is increased once the *P. aeruginosa* ST274 have been well reported as cause of respiratory infections of cystic fibrosis patients in Europe (Oliver et al., 2015). Again, since this high-risk clone and carbapenem-resistance are currently associated to healthcare settings, and that the UP4 *P. aeruginosa* was selected in a red deer from a humanized area, maybe the environmental context was determinant in the spread of this high-risk carbapenem-resistant *P. aeruginosa* clone by indirect contamination but further epidemiological studies are needed to better understand the transmission dynamics. The relation of high-risk clones and MDR in *P. aeruginosa* is higher, since the disseminated high-risk clones usually show an AMR profile. On the other contrary, tend to be susceptible to antibiotics (Mulet et al., 2013).

Wild birds have been highlighted as potential reservoirs and disseminators of pathogens, including *P. aeruginosa* (Rodrigues et al., 2021; Sharma et al., 2014). The results highlight that among wild ungulates studied, particularly red deer can act as potential carbapenem-resistant *P. aeruginosa* reservoirs increasing the human health and environmental risk, as their urine and faeces will contaminate the soil and contribute to the dissemination of AMR to/from humans, animals and the environment (Ruiz-Roldán et al., 2020). Their spatial and ecological flexibility places them as potential epidemiological vectors of *P. aeruginosa* and associated resistance mechanisms.

Table 2
Antimicrobial resistance and typing characterization of carbapenem-resistant *Pseudomonas aeruginosa* isolated from red deer.

Bacterial typing	Sequence type	ST274
	Serogroup	O3
Antimicrobial resistance	Resistance profile	R-IMI, I-MRP, I-DOR
	ECOFF non-wildtype profile	IMI, MRP, DOR
	MIC (μg/mL)	IMI (12), MRP (6)
	Antimicrobial resistance genes	bla_{PAO}, bla_{PDC-24}, $bla_{OXA-486}$, aph (3′)-IIb, fosA, catB7

MIC – Minimal inhibitory concentration; R – resistant profile; I – Intermediate resistant profile; IMI – Imipenem; MRP – Meropenem; DOR – Doripenem.

5. Conclusions

Environment, and particularly wildlife, have been underlooked on the monitoring of AMR bacteria and resistance determinants, particularly carbapenem-resistant *Pseudomonas* spp. To the best of our knowledge, this is the first evidence to describe carbapenem-resistant *P. aeruginosa* in a widespread deer species in Europe, suggesting the important role of wild ungulates on the ongoing spread of carbapenem-resistant *P. aeruginosa*, with potential epidemiological implications. While *P. aeruginosa* is a ubiquitous pathogen broadly detected in the environment, our findings provide novel insights into the current knowledge of *P. aeruginosa* in wildlife, setting the basis for future surveillance studies including wild ungulates in other European countries, to assess other possible reservoirs of MDR bacteria and resistance determinants, as these species are increasing in number and distribution.

CRediT authorship contribution statement

Rita Tinoco Torres: Conceptualization, Writing – original draft, Supervision, Funding acquisition, Project administration. **Mónica V. Cunha:** Writing – review & editing. **Helena Ferreira:** Resources. **Carlos Fonseca:** Resources. **Josman Dantas Palmeira:** Methodology, Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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