Assessing the Occurrence of Carbapenemase Producers Using Marine Animals as Sentinel Species

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Abstract

Carbapenemase-producing (CP) strains represent a substantial global threat, deactivating carbapenems and conferring resistance to β-lactam antibiotics. They can spread across various environments, yet data on their presence in marine animals are sparse. This study aimed to assess the occurrence of carbapenemase-producing strains in wild marine animals and to analyse their antimicrobial resistance (AMR) profiles to crucial antimicrobials and heavy metals frequently encountered in the marine environment due to anthropogenic activity. A total of 28 samples were obtained from a fish auction in the Centre Region of Portugal. Non-fermenting bacilli (NFB) was isolated from the visceral content of wild marine animals. Identification of isolates was achieved through PCR-based amplification of the 16S rRNA gene, followed by sequencing. Antimicrobial susceptibility testing was conducted according to EUCAST guidelines, covering nine antimicrobials. Research of carbapenemases and metal tolerance genes was conducted by PCR, and statistical analysis utilized the Fisher's exact test. *Pseudomonas* spp. and *Aeromonas* spp., among other isolates were identified (*n*=47/9/7, respectively). Susceptibility profiles showed 100% resistance or intermediate resistance to ticarcillin, piperacillin, piperacillin-tazobactam, ceftazidime, ciprofloxacin, and imipenem (*n*=63/63), while 27% were resistant to meropenem (*n*=17/63) and 13% to tobramycin (*n*=8/63). All of them exhibited susceptibility to amikacin and carried

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multidrug resistance (MDR) profiles, including heavy metal genes (*merA* and *silA*). None harboured the carbapenemase genes searched (bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{NDM} or/and bla_{VIM}). In this study, MDR profiles to clinically important antimicrobials were observed, including to carbapenems. However, no carbapenemase-producing strains were identified, suggesting the presence of other genes or alternative mechanisms of resistance. These findings underscore the importance of monitoring AMR in marine ecosystems, particularly given its close ties to the food chain.

Key words: *carbapenemases; multidrug-resistance; antimicrobials; heavy-metals; marine animals; Pseudomonas spp.; Aeromonas spp.*

Introduction

Antimicrobial resistance (AMR) has become internationally recognised as a significant global health challenge, and has the potential to disseminate across human, animal, and environmental domains, resulting in amplified human morbidity and mortality (WHO 2017; ECDC, 2022).

Over the last decade, the emergence and prevalence of carbapenemase-producing strains have been growing at a global scale and are directly linked to a risk factor for nosocomial infections. This pressing concern has led to their inclusion in the roster of high-priority pathogens, demanding urgent Research and Development endeavours for novel antimicrobials (WHO, 2017). Furthermore, carbapenemase-producing strains have recurrently been associated with the emergence of Multidrug-Resistant (MDR) clones, posing a substantial threat given the limited efficacy of the available antimicrobials (Nordmann and Poirel, 2019). As reported by the European Centre for Disease Prevention and Control, the highest levels of AMR in southern and eastern Europe were reported in 2020. This underscores the critical significance of early detection and identification of these strains (ECDC, 2022).

Carbapenemases are enzymatic proteins capable of hydrolysing carbapenem compounds along with other β-lactams, which stand out as the prevailing mechanisms behind carbapenem resistance. Notably, these enzymes have been associated with an easy spread into the community and environmental settings, mainly accomplished through mobile genetic elements, such as plasmids or transposons (Nordmann and Poirel, 2019). Such mechanisms foster horizontal gene transfer among pathogens, potentially leading to the proliferation of AMR within other bacterial ecosystems, including marine environments (Bonardi and Pitino 2019; Chen et al., 2020; Dewi et al., 2020; Duff et al., 2020; Norman et al., 2021).

Nonetheless, other mechanisms of resistance to carbapenems may also be documented. These include the depletion of outer membrane porins (e.g., *Enterobacterales* carrying extended-spectrum beta-lactamases or AmpC enzymes), as well as the loss of OprD porins expression in *Pseudomonas aeruginosa*. Furthermore, resistance might stem from the presence of efflux pumps or even due to mutations in penicillin-binding proteins (e.g., *Escherichia coli, P. aeruginosa*, and *Acinetobacter baumannii*) (Botelho et al., 2019; Nordmann and Poirel, 2019).

Currently, some of the most prevalent carbapenemases found in non-fermenting bacilli encompass different Ambler classes: A - *Klebsiella pneumoniae* carbapenemase (KPC) and Guiana extended-spectrum-ß-lactamases (GES); B – Verona integron-mediated metallo-ß-lactamases (VIM), Active-on-imipenem (IMP)

and New Delhi metallo-ß-lactamases (NDM), as well as class D-oxacillinases (OXA-48) (Bogaerts et al., 2013; Nordmann and Poirel 2019). The escalation of selective pressure on bacteria finds its roots in the improper or excessive use of antimicrobials in both human and animal contexts. However, this rise in selective pressure may also be exacerbated by the presence of heavy metals within the environment (Rebelo et al., 2021). Available data have underscored the potential role of metal tolerance in contributing to the emergence of MDR bacteria, primarily through an indirect selection process. This phenomenon arises due to the co-location of genes responsible for both metal and antibiotics tolerance/resistance within the same mobile genetic elements, such as plasmids (Romero et al., 2017; Figueiredo et al., 2019).

Heavy metals naturally exhibit a widespread presence in marine ecosystems. However, it is primarily anthropogenic activities that serve as the principal contributors, emanating from sources like metal-laden food additives, agricultural practices related to biosecurity and hygiene, industrial and mining operations, as well as wastewater discharges (Tappin et al., 2010; Romero et al., 2017; Rebelo et al., 2021). Scientific data has shown that wild marine animals, even in the absence of direct antimicrobial treatment, could serve as potential reservoirs for resistant bacterial strains capable of infecting or colonising other animals or humans (Wallace et al., 2013; Marti et al., 2018; Duff et al., 2020; Norman et al., 2021). Undoubtedly, wild marine animals possess the capacity to accumulate heavy metals, a factor that could potentially facilitate the acquisition of AMR genes and lead to the emergence of a reservoir of MDR bacteria, including carbapenemase-producing strains. This scenario poses a significant threat to the safety and quality of marine water, consequently endangering public health (Woodford et al., 2018; Botelho et al., 2019; Figueiredo et al., 2019). The point is emphasised that AMR has been scarcely documented in wild marine animals, highlighting their crucial role as sentinels of marine well-being and health (Norman et al., 2021).

This study seeks to provide novel perspectives on the prevalence of AMR, with emphasis on carbapenemase-producing organisms in wild marine animals along the central Portuguese coast, to assess the impact of anthropogenic activities on their potential dispersion within the marine ecosystem.

Methods

Study design

Between May and April 2022, a total of 28 samples were collected from visceral tissues of wild marine fishes: *Trachurus trachurus* (*n*=2), *Dicentrarchus labrax* (*n*=1), *Trisopterus luscus* (*n*=1), *Zeus faber* (*n*=8), *Diplodus sargus* (*n*=6), *Merluccius merluccius* (*n*=4), *Todarodes sagittatus* (*n*=3), *Scyliorhinus canicula* (*n*=2) and *Raja brachyura* (*n*=1). All fish were captured within the Northern Atlantic waters of Portugal's Central Region. The specimens were conveyed within a regulated refrigerated setting and expeditiously subjected to processing upon their reception at the microbiology laboratory. This procedural sequence entailed the extraction of visceral contents, followed by buffered peptone water pre-enrichment. Subsequently, the samples were subjected to a homogenisation process (Interscience® BagMixer®), followed by overnight incubation for 16-24 hours at 35±2°C.

Bacterial selection and growing conditions

For the selection of the carbapenemase-producing strains, the samples were plated onto ChromID® Carba Smart Agar (Biomerieux), and incubated for 18-24 hours at 35±2°C. The selection of representative colonies was chosen according to Woodford et al. (2018). Distinctive colonies were selected and cultivated on MacConkey Agar (Oxoid), followed by isolation on Trypticase Soy Agar (Liofilchem) to achieve a state of pure culture.

Identification of isolates

The identification of representative and suspected carbapenemase-producing strains was accomplished using the amplification and sequencing of the 16S rRNA gene as previously described (Héritier et al., 2003). Sequencing was conducted by Eurofins (https://www.eurofins.pt/), and the analysis was carried out using the NCBI Reference Sequence Database and the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov).

Study of antimicrobial susceptibility

Antimicrobial susceptibility testing was conducted using the standard disk diffusion method on Mueller Hinton agar (Biolab®), following the guidelines outlined by the European Committee on Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing, 2022). This process involved the assessment of nine antimicrobials: piperacillin (PRL, 30 μg), piperacillin-tazobactam (TZP, 30-6 μg), ticarcillin (TIC, 75 μg), ceftazidime (CAZ, 10 μg), imipenem (IMP, 10 μg), meropenem (MEM, 10 μg), ciprofloxacin (CIP, 5 μg), amikacin (AK, 30 μg) and tobramycin (TOB, 10 μg) (Oxoid®). MDR profiles were deemed when bacteria exhibited resistance to at least three or more antimicrobials from different categories (Magiorakos et al., 2012).

Study of carbapenemases and heavy metal susceptibility

Isolates resistant to carbapenems and suspected to be carriers of the most prevalent carbapenemases were selected for PCR screening for bla_{GES}, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC} genes as previously described (Table 1) (Bogaerts et al., 2013).

Genes linked to bacterial tolerance of heavy metals were chosen according to their potential occurrence in marine environment and subsequently probed through PCR: *pcoA* (periplasmic multicopper oxidase), *pcoD* (copper inner membrane pump); *silA* (efflux pump), *silE* (periplasmic silver-sequestration protein); *arsB* (efflux pump); and *merA* (mercuric reductase) (Table 1) (Mourão et al., 2015).

Results

Bacterial selection

Colonies growing on ChromID® Carba Smart Agar displayed morphological characteristics that did not align with the descriptions provided in the manufacturer's instructions. Consequently, the selected isolates predominantly appeared colourless (*n*=47), while a smaller number exhibited shades of brown (*n*=9), red (*n*=2), and green/greenish (n=5) colouration.

Identification of isolates

Of all the representative isolates (*n*=56), identification was performed through sequencing of the 16S rRNA gene. These isolates were categorised into *Pseudomonas* spp. (*n*=47, colourless colonies) and *Aeromonas* spp. (*n*=9, brownish colonies). The remaining colonies, exhibiting varying shades of red and greenish tones, were categorised as non-fermenting bacilli (NFB) (*n*=7) and were excluded from sequencing.

Table 1. Primer sets for carbapenemase and heavy metal tolerance genes research

Target gene	Primers	Primer sequences (5'-3')	Amplicon size (bp)	Annealing (C°)	References
16S rRNA	FW	AGAGTTTGATCHTGGYTYAGA	1465	50	Héritier et al., 2003
	RV	ACGGYTACCTTGTTACGACTTC			
bla$_{KPC}$	FW	TCGCCGTCTAGTTCTGCTGTCTTG	353	60	Bogaerts et al., 2013
	RV	ACAGCTCCGCCACCGTCAT			
bla$_{GES}$	FW	CTGGCAGGGATCGCTCACTC	600	57	Bogaerts et al., 2013
	RV	TTCCGATCAGCCACCTCTCA			
bla$_{VIM}$	FW	TGTCCGTGATGGTGATGAGT	437	61	Bogaerts et al., 2013
	RV	ATTCAGCCAGATCGGCATC			
bla$_{IMP}$	FW	ACAYGGYTTRGTDGTKCTTG	387	57	Bogaerts et al., 2013
	RV	GGTTTAAYAAARCAACCACC			
bla$_{NDM}$	FW	ACTTGGCCTTGCTGTCCTT	603	57	Bogaerts et al., 2013
	RV	CATTAGCCGCTGCATTGAT			
pcoA	FW	CTCGCGGATGTCAGTGGCTACACCT	504	60	Mourão et al., 2015
	RV	ATCCGGAAGGTCAGCACCGTCCATAGAC			
pcoD	FW	CTGGCCACACTTGCCTGGGG	500	55	Mourão et al., 2015
	RV	CACGCTACGGCGCCCAGAAT			
silA	FW	GCAAGACCGGTAAAGCAGAG	936	59	Mourão et al., 2015
	RV	CCTGCCAGTACAGGAACCAT			
silE	FW	GTTCGTCATGGTYTCATGAGC	264	62	Mourão et al., 2015
	RV	GTACTYCCCCGGACATCACTAATT			
merA	FW	ACCATCGGCGGCACCTGCGT	1238	65	Mourão et al., 2015
	RV	ACCATCGTCAGGTAGGGGAAC			
arsB	FW	AGTGAAAGACAGACGAAGCG	849	60	Mourão et al., 2015
	RV	GGCAGATAGTGTGGAATGCG			

Legend: FW – Forward; RV – Reverse

Study of antimicrobial susceptibility

The antimicrobial susceptibility testing results revealed resistance or intermediate resistance among the isolates to all β-lactams, including subclasses such as penicillins, cephalosporins, and carbapenems, as well as to fluoroquinolones. Among the 63 isolates obtained from the visceral samples of wild marine fish, 100% (*n*=63/63) exhibited resistance or

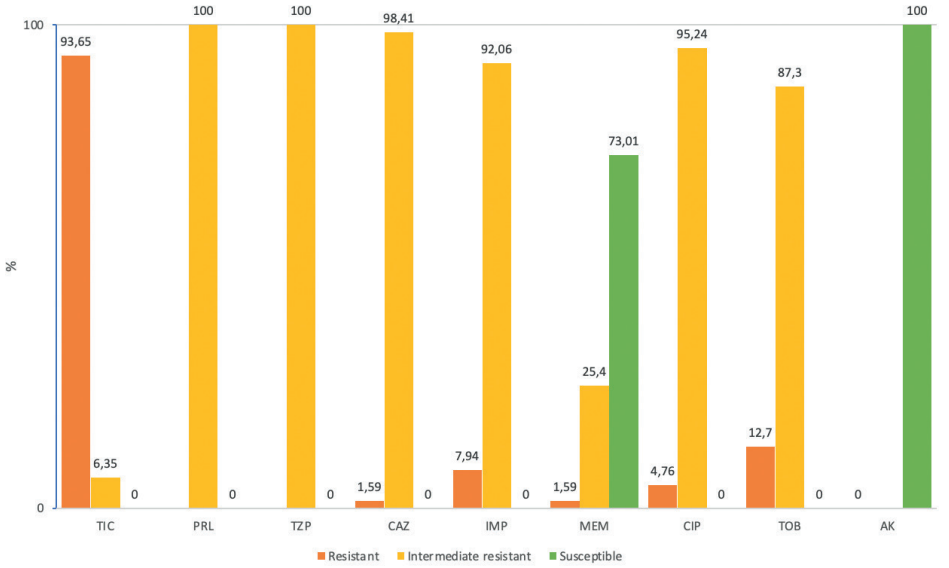

Figure 1. Distribution of antimicrobial resistance among the bacterial collection

Legend: $P<0.05$ (Fisher's exact test) Ticarcillin (TIC), piperacillin (PRL), piperacillin-tazobactam (TZP), ceftazidime (CAZ), imipenem (IMP), tobramycin (TOB), amikacin (AK), meropenem (MEM), ciprofloxacin (CIP)

intermediate resistance to TIC, PRL, TZP, CAZ, IMP, and CIP, followed by MEM (27%, *n*=17/63: *Pseudomonas* spp., *n*=16; *Aeromonas* spp., *n*=1), and TOB (13%, *n*=8/63: *Aeromonas* spp., *n*=3; other NFB, *n*=3 and *Pseudomonas* spp., *n*=2). Notably, all isolates demonstrated susceptibility to AK (Figure 1).

The findings unveiled seven distinct MDR profiles (heavy metal tolerance genes were included) (Table 2). Noteworthy, 61.9% of the isolates shared the MDR profile "TIC, PRL, TZP, CAZ, IMP and CIP" (*n*=32/63: *Pseudomonas* spp., *Aeromonas* spp., and NFB; *n*=22/6/4, respectively), followed by "TIC, PRL, TZP, CAZ, IMP, MEM and CIP" (*n*=10/63: Pseudomonas spp., *n*=10), "TIC, PRL, TZP, CAZ, IMP, TOB and CIP" (n=7/63: Pseudomonas spp., Aeromonas spp., and NFB, *n*=2/2/3, respectively). Lastly, the final profile was identified in just one isolate of *Aeromonas* spp. and exhibited resistance to all tested antimicrobial families, except for amikacin (penicillins, cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones) (Figure 2).

Additionally, among the entire isolate pool, 9.5% (*n*=6/63) and 6.4% (*n*=4/63) of *Pseudomonas* spp. were found to harbour the *merA* gene within two distinct MDR profiles: i) 'TIC, PRL, TZP, CAZ, IMP, and *merA* or ii) 'TIC, PRL, TZP, CAZ, IMP, MEM, CIP, and *merA*. Additionally, the gene *silA* was identified in a sole profile, constituting 4.8% (*n*=3/63) of the isolates, characterised as TIC, PRL, TZP, CAZ, IMP, CIP, and *silA*" (Table 2, Figure 2).

Study of carbapenemase and heavy metal susceptibility

After conducting a PCR-based screening to detect the presence of carbapenemase

Table 2. Characterisation of multidrug-resistant profiles among the collection of isolates from wild marine animals

Profiles	Antibiotics	HMTG	Isolates
I	TIC, PRL, TZP, CAZ, IMP, CIP	—	*Pseudomonas* spp (22) *Aeromonas* spp (6) Others NFB (4)
II	TIC, PRL, TZP, CAZ, IMP, CIP	*merA*	*Pseudomonas* spp (4)
III	TIC, PRL, TZP, CAZ, IMP, CIP	*silA*	*Pseudomonas* spp (3)
IV	TIC, PRL, TZP, CAZ, IMP, MEM, CIP	—	*Pseudomonas* spp (10)
V	TIC, PRL, TZP, CAZ, IMP, TOB, CIP	—	*Pseudomonas* spp (2) *Aeromonas* spp (2) Others NFB (3)
VI	TIC, PRL, TZP, CAZ, IMP, MEM, CIP	*merA*	*Pseudomonas* spp (6)
VII	TIC, PRL, TZP, CAZ, IMP, TOB, MEM, CIP	—	*Aeromonas* spp (1)

Legend: HMTG – Heavy Metal Tolerance genes; Ticarcillin (TIC), piperacillin (PRL), piperacillin-tazobactam (TZP), ceftazidime (CAZ), imipenem (IMP), tobramycin (TOB), meropenem (MEM), ciprofloxacin (CIP); isolates showing intermediate susceptibility were categorised as resistant

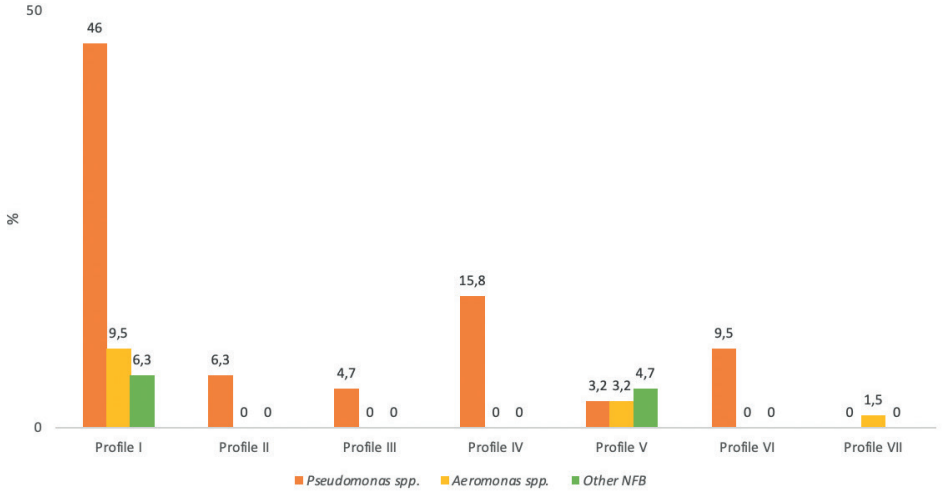

Figure 2. Distribution of the different multidrug-resistant profiles, including heavy metal tolerance genes, among different genera or bacterial groups

Legend: Profile I: TIC, PRL, TZP, CAZ, IMP, CIP (*n*=32/63); **Profile II:** TIC, PRL, TZP, CAZ, IMP, CIP, *merA* (*n*=4/63); **Profile III:** TIC, PRL, TZP, CAZ, IMP, CIP, *silA* (*n*=3/63); **Profile IV:** TIC, PRL, TZP, CAZ, IMP, MEM, CIP (*n*=7/63); **Profile V:** TIC, PRL, TZP, CAZ, IMP, TOB, CIP (*n*=10/63); **Profile VI:** TIC, PRL, TZP, CAZ, IMP, MEM, CIP, *merA* (*n*=6/63); **Profile VII:** TIC, PRL, TZP, CAZ, IMP, TOB, MEM, CIP (*n*=1/63). Isolates showing intermediate susceptibility were categorised as resistant

genes (bla_{KPC}, bla_{GES}, bla_{IMI}, bla_{NDM} or bla_{VIM}) in isolates exhibiting resistance to carbapenems (*Pseudomonas* spp., *Aeromonas* spp., and others NFB: *n*=3/2/1, respectively), it became evident that none of the isolates harboured these carbapenem resistant genes.

In the search for metal tolerance genes, it was noted that 15.9% (*n*=10/63) of *Pseudomonas* spp. carried the *merA* gene, while 4.8% (*n*=3/63) exhibited the *silA* gene. The presence of *silE*, *pcoA*, *pcoD*, and *arsB* genes, responsible for conferring tolerance to silver, copper, and arsenic, was not observed.

Discussion

The culture media ChromID® Carba Smart Agar is made to select *Enterobacterales* carbapenemase-producing from human clinical specimens. Using samples from the marine environments, as in this study, the conditions of growth may vary, since the bacterial strains may have other individual requirements. Nevertheless, it allowed for the recovery of a high rate of carbapenem-resistant or intermediated-resistant isolates. Following the manufacturer's instructions, the emergence of other MDR bacteria is possible, which could explain the growth of *Pseudomonas* spp., *Aeromonas* spp., and other NFB instead of *Enterobacterales*. Indeed, the significant occurrence of *Pseudomonas* spp. observed in this study may be linked to their inherent presence within the microbiota of carnivorous, omnivorous, and planktivorous fish. This prevalence is further reinforced by their widespread distribution across aquatic environments (Egerton et al., 2018).

Despite scientific literature documenting that wild marine life can be an important reservoir of AMR genes and could play a significant role in their worldwide dissemination, especially through migratory animals, this is the first study to report high levels of resistance or intermediate resistance to critical or highly important antimicrobials in wild marine fish (Marti et al., 2018; Chen et al., 2020; Fernandes et al., 2021; Norman et al., 2021). High resistance (R) or intermediate resistance (IR) rates to aminopenicillins with or without beta-lactamase inhibitors, cephalosporins, fluoroquinolones, carbapenems, and aminoglycosides (TIC-R: 93.65%, TIC-IR: 6.35%; PRL-IR and TZP-IR: 100%; CAZ-R: 1.59%, CAZ-IR:98.41%; CIP-R: 4.76%, CIP-IR:95.24%; IMP-R: 7.94%, IMP-IR:92.06%; MEM-R: 1.59%, MEM-IR:25.4%; TOB-R:12.7%, TOB-IR:87.3%) were observed. A few published studies on sea turtles, pinnipeds, shrimps and mussels described low levels of resistance or intermediate resistance to aminoglycosides, fluoroquinolones, or carbapenems. However, *Pseudomonas* spp. or *Aeromonas* spp. was poorly reported (Fernandes et al., 2021; Norman et al., 2021; Celik et al., 2023). In contrast, another study documented a considerable abundance of *Pseudomonas* spp. along with a heightened resistance rate to aminoglycosides (Wallace et al., 2013). When comparing the outcomes of our investigation to those of other studies, alongside the existing data on contamination in freshwater sources such as rivers, sewage, and seawater, a noteworthy pattern emerged: the existence of carbapenemase-producing organisms and a substantial prevalence of metal tolerance genes (66.6%) (Dewi et al., 2020; Gambino et al., 2022; Montezzi et al., 2015).

Scientific data has reported that carbapenem resistance has primarily been detected in human-pathogenic *Enterobacterales* (Nordmann and Poirel, 2019). Nevertheless, this resistance phenomenon goes beyond the confines of this bacterial group, encompassing a wide array of

other Gram-negative families (Dias et al., 2014; Botelho et al., 2019). Notably, this includes various species of *Pseudomonas* carbapenemases-producing strains, or *Aeromonas* spp. originating from the marine ecosystem. (Montezzi et al., 2015; Figueras Salvat and Ashbolt, 2019; Dewi et al., 2020).

A concerning aspect is the carbapenems-resistant genes be located within mobile genetic elements, enabling their transfer between genera or species. This phenomenon has already been observed in *Aeromonas salmonicida*, which demonstrates a natural exchange of genetic content with *Pseudomonas* spp. and enteric plasmid types (Botelho et al., 2019). This is an exceedingly unsettling factor, as it facilitates the dissemination of these genes across diverse environments, including the food chain or terrestrial wild animals (Dias et al., 2014; Romero et al., 2017; Figueras Salvat and Ashbolt, 2019).

The findings from our study indicate that none of the carbapenem-resistant isolates analysed exhibited the presence of the most prevalent carbapenemase genes that were investigated. Nonetheless, it is important to note that other genes or resistance mechanisms may potentially be at play (Nordmann and Poirel, 2019). Indeed, alternative resistance mechanisms have been documented in *Pseudomonas* spp. These encompass the depletion of outer membrane porins, the suppression of OprD porin expression, the upregulation of genes encoding efflux pumps, or any alterations that affect the production level or binding affinity of penicillin-binding proteins and mechanisms (Botelho et al., 2019; Nordmann and Poirel, 2019; Dewi et al., 2020).

Additionally, low rates of heavy metal tolerance genes, *silA* and *merA*, were identified in our study. Notably, these occurrences were exclusive to the *Pseudomonas* genus, showing that wild marine fish could be a source for the co-selection of heavy metals with bacteria resistant to antibiotics (Norman et al., 2021; Fulham et al., 2022). Moreover, marine organisms display susceptibility to the bioaccumulation of mercury, exhibiting an inclination to amass elevated levels, particularly among predatory fish. This heightened accumulation propensity contributes to an increased potential for the proliferation of bacteria that possess tolerance to heavy metals. Also, silver is present naturally in the aquatic environment, but at very low concentration, and the identification of the *silA* gene has been established as an indicative marker of anthropogenic pollution, particularly emanating from wastewater discharges (Tappin et al., 2010). However, reporting on the silA operon and mercuric reductase gene as contributors to silver or mercury tolerance in *Pseudomonas* spp. remains scant.

It has also been demonstrated that the mechanisms employed by mercury-tolerant bacteria could potentially play a role in mitigating mercury pollution. This is achieved through the conversion of more toxic forms of mercury into less harmful variants (Zhang et al., 2012). Nonetheless, this does not diminish apprehensions surrounding the observation that genes present in both AMR are frequently situated on mobile genetic elements like transposons or plasmids. Such positioning facilitates the more accessible spread of these resistances through the process of horizontal gene transfer (Zhang et al., 2012; Romero et al., 2017; Rebelo et al., 2021).

Some authors advocate the importance of monitoring AMR genes in wild marine animals, since it can be an indicator of the pollution state of the marine environments (Bonardi and Pitino, 2019; Chen et al., 2020). A matter of concern revolves around the possibility that the marine en-

vironment could serve as a silent origin of AMR, posing a potential risk to human health. This situation could engender the colonisation of the human gut through seafood consumption, and further extend to the potential emergence of occupational diseases among individuals involved in activities such as seafood handling or other endeavours within marine settings (Marti et al., 2018; Figueras Salvat and Ashbolt, 2019).

Our findings underscore wild marine animals as reservoirs for MDR *Pseudomonas* spp, followed by *Aeromonas* spp. This phenomenon is particularly pronounced in relation to resistance against the highest priority critically important antimicrobials, suggesting that wild marine animals may serve as latent and potentially significant hotspots for the diversification of bacterial populations.

To effectively address this global concern, the implementation of rigorous monitoring and systematic surveillance within marine environments is imperative. This strategy is vital to attain a thorough comprehension of the existing state of AMR within marine ecosystems, encompassing not only the presence of carbapenemases or alternative mechanisms of carbapenem resistance, but also the occurrence of heavy metal tolerance genes. Undoubtedly, the investigation of wild marine animals could offer invaluable insights into the assessment of pollution levels within marine ecosystems, thereby giving a strong contribution to food safety and public health.

Ethical approval

The animals were procured from a fish market in adherence to the protocol approved by the Ethics Committee of the University School Vasco da Gama, Coimbra, Portugal (Reference No. 09/2022). This study was conducted as part of the project titled "Marine animals as sentinels to evaluate spread of carbapenemase producers." Data containing confidential information were excluded to ensure compliance with Regulation (EU) 2016/679. Principal Investigator: Prof. Eduarda Silveira, PhD.

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Procjena pojavnosti proizvođača karbapanemaza uporabom morskih životinja kao pokusne vrste

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Sojevi koji proizvode karbapanemaze (CP) predstavljaju znatnu globalnu prijetnju, deaktivirajući karbapeneme i pružajući otpornost na β-laktamske antibiotike; mogu se širiti kroz razna okruženja, a ipak, podatci o njihovoj prisutnosti u morskim životinjama su rijetki. Ova je studija za cilj imala procijeniti pojavnost sojeva koji proizvode karbapanemaze u divljih morskih životinja i analizirati njihove profile antimikrobne otpornosti (AMR) na glavne antimikrobne lijekove i teške metale koji se često pronalaze u morskom okruženju zbog antropogene aktivnosti. Na aukciji ribe u centralnoj regiji Portugala prikupljeno je ukupno 28 uzoraka. Nefermentirajući bacili (NFB) su izolirani iz visceralnog tkiva divljih morskih životinja, uporabom ChromID® Carba Smart agara nakon predobogaćivanja. Identifikacija

izolata postignuta je pojačanjem 16S rRNK gena na bazi PCR-a, nakon čega je uslijedilo sekvenciranje. Ispitivanje prijemčivosti na antimikrobne lijekove provedeno je prema EUCAST smjernicama, pokrivajući devet antimikrobnih lijekova. Istraživanje karbapanemaza i gena tolerancije na metale provedeno je pomoću PCR-a, a statistička analiza je rabila Fisherov egzaktni test (GRAPHPAD Prisma® softver, verzija 8.4.2). Među ostalim izolatima (*n*=47/9/7) pronađene su *Pseudomonas* spp. i *Aeromonas* spp. Profili prijemčivosti pokazali su 100%-tnu otpornost ili srednju otpornost na: tikarcilin, piperacilin, piperacilin-tazobaktam, ceftazidim, ciprofloksacin i imipenem (*n*=63/63), 27 % ih je bilo otporno na meropenem (*n*=17/63) i 13 % na tobramicin (*n*=8/63), a svi su pokazali prijemčivost na amikacin i nosili su profile otpornosti na višestruke lijekove (MDR), uključujući gene za otpornost na teške metale (*merA* i *silA*). Niti jedan nije imao tražene gene karbapanemaze (bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{NDM} ili/i bla_{VIM}). U ovoj su studiji istraživani profili MDR na klinički važne antimikrobne lijekove, uključujući karbapanemaze. Međutim, sojevi koji proizvode karbapanemaze nisu identificirani, što ukazuje na prisutnost drugih gena ili alternativnih mehanizama otpornosti. Ovi nalazi naglašavaju važnost praćenja otpornosti na antimikrobne lijekove u morskim ekosustavima, posebno s obzirom na njihovu usku povezanost s prehrambenim lancem.

Ključne riječi: *karbapanemaze, otpornost na višestruke lijekove, antimikrobni lijekovi, teški metali, morske životinje, Pseudomonas spp., Aeromonas spp.*