ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Marine animals as sentinels to evaluate spread of carbapenemases producers

Marine Denise Kameneff

Coimbra, (Julho 2022)

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“The heart of man is very much like the sea, it has its storms, it has its tides and in its depths it has its pearls too.”

Vincent van Gogh

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ABBREVIATIONS LIST

AK - Amikacin

AMR – Antimicrobial resistant/resistance

β - Beta

CAZ - Ceftazidime

CIP - Ciprofloxacin

CP - Carbapenemase-producing

CPE - Carbapenemase-producing *Enterobacterales*

ECDC - European Center for Disease Prevention and Control

e.g. - *ergo gratia*

ESBL - Extended-spectrum beta-lactamases

EUCAST - European Committee on Antimicrobial Susceptibility Testing

GES - Guiana extended-spectrum β-lactamases

GNB - Gram-negative Bacillis

IMP - Imipenem

KPC - *Klebsiella pneumoniae* carbapenemase

MA - Marine animals

MDR - Multidrug-Resistant

MEM - Meropenem

NDM - New Delhi Metallo-β-lactamases

NFB - Non-fermenting Bacillis

OXA - Oxacillinase

PCR - Polymerase chain reaction

PRL - Piperacillin

RNA - Ribonucleic Acid

TIC - Ticarcillin

TOB - Tobramycin

TZP - Piperacillin-tazobactam

VIM - Verona integron-mediated Metallo-β-Lactamases

Marine animals as sentinels to evaluate spread of carbapenemases producers

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RESUMO

Estirpes portadoras de carbapenemases (CP) constituem uma grande preocupação a nível mundial, em virtude de inativarem os carbapenemos, e conferirem resistência não só a estes, que são o último recurso para infeções nosocomiais, mas também a outros β-lactâmicos. As carbapenemases podem disseminar-se entre diferentes Géneros e Famílias e têm sido reportadas não só em estirpes responsáveis por infecções nosocomiais, mas também em outros ambientes. Contudo, em animais marinhos (MA) os dados são escassos. Este estudo teve por objectivo monitorizar a prevalência de estirpes CP ou resistência a outros antimicrobianos incluindo metais pesados em MA provenientes da Costa portuguesa.

Obtiveram-se 28 amostras de MA, numa lota de peixe da Região Centro de Portugal. A partir do conteúdo visceral, com auxílio da técnica de pré-enriquecimento, procedeu-se à seleção de bacilos de gram-negativo (GNB) Não-Fermentadores (NFB) e CP com recurso ao meio de cultura ChromID Carba Smart Agar® e prova da oxidase. A identificação dos isolados foi realizada através da amplificação do gene 16S rRNA por PCR (Polimerase Chain Reaction) e sequenciação. O estudo do perfil de suscetibilidade incluiu nove antimicrobianos e realizou-se de acordo com as normas de referência europeias; a pesquisa de carbapenemases e genes de tolerâncias a metais foi realizada por PCR. Procedeu-se à análise estatística aplicando o teste exato de Fisher e recorrendo ao software GRAPHPAD Prisma® (versão 8.4.2).

Foram selecionados 63 NFB: *Pseudomonas* spp., *Aeromonas* spp. e outros NFB (n=47/9/7, respetivamente). Quanto ao perfil de resistência observou-se que: 100% eram resistentes à ticarcilina, piperacilina, piperacilina-tazobactam, ceftazidima, ciprofloxacina e imipenemo (n=63/63); 27% ao meropenemo (n=17/63); 13% à tobramicina (n=8/63: *Aeromonas* spp., *Pseudomonas* spp e NFB; n=3/2/3, respetivamente); todos apresentaram suscetibilidade à amicacina. Nenhum dos seis isolados suspeitos de serem PC (*Pseudomonas* spp., *Aeromonas* spp. e NFB: n=3/2/1, respetivamente) eram portadores de genes codificadores das carbapenemases *bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{IMP}$, *bla*$_{NDM}$ ou *bla*$_{VIM}$. Os genes *mer*A (n=10/63) e *sil*A (n=3/63), representaram uma prevalência de 15,87% e 4,76%, respetivamente. Verificou-se que todos os isolados apresentaram perfil de MDR.

Os MA são portadores de bactérias MDR, mas não de estirpes CP. Os resultados realçam a necessidade de monitorizar a AMR em ambiente marinho, particularmente no que respeita à estreita ligação com a cadeia alimentar.

Palavras-chave: cabapenemases, metais pesados, multiresistência, antimicrobianos, animais marinhos.

ABSTRACT

The carbapenemase-producing (CP) strains are a great concern worldwide, due to their capacity to inactivate the carbapenems, and conferring resistance not only to this group which is one of the last-resort antibiotics for nosocomial infections, but also to other groups of β-lactams. The carbapenemases can be spread between various Genus and Families and have not only been reported, in nosocomial infections, but also in other environments. However, the available data in marine animals (MA) are scarce. This study aimed to monitor the prevalence of CP strains or resistance to other antimicrobials.

Twenty-eight samples were recovered from MA, from a fish auction in the Centre region of Portugal. From the visceral content, with the aid of the pre-enrichment technique, CP gram-negative bacilli (GNB) Non-Fermenting (NFB) were selected using the ChromID Carba Smart Agar® culture medium and oxidase test. The isolates identification was performed by the amplification of the 16S rRNA gene by PCR (Chain Reaction Polymerase) and sequencing. The study of the susceptibility profile included nine antimicrobials and was carried out according to the European reference standards; research of carbapenemases and metal tolerance genes were performed by PCR. The statistical analysis was performed using the Fisher's exact test and GRAPHPAD Prisma® software (version 8.4.2).

Sixty-Three NFB were recovered: *Pseudomonas* spp., *Aeromonas* spp. and other NFB (n=47/9/7, respectively). As for the resistance profile, it was observed: 100% for ticarcillin, piperacillin, piperacillin-tazobactam, ceftazidime, ciprofloxacin and imipenem (n=63/63); 27% to meropenem (n=17/63); 13% to tobramycin (n=8/63: *Aeromonas* spp., *Pseudomonas* spp. and NFB; n=3/2/3, respectively); all were susceptible to amikacin. In none of the six representative isolates suspected of being CP (*Pseudomonas* spp., *Aeromonas* spp. and NFB, n=3/2/1, respectively) were observed bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{NDM} or/and bla_{VIM} encoding carbapenemases genes. The *merA* (n=10/63) and *silA* genes (n=3/63) represented a prevalence of 15,9% and 4,8%, respectively. It was noted that all the isolates carried a Multidrug-Resistant (MDR) profile.

The MA harbour bacteria MDR, but not CP strains, thus allows to conclude that carbapems resistance was mediated by other mechanisms. The results highlight the need to monitor the antimicrobial resistance in the marine environment, particularly concerning the close connection with the food chain.

Keywords: carbapenemases, heavy metals, multi drug resistant, antimicrobials, marine animals.

BACKGROUND

The increase of antimicrobial resistance (AMR) is a global health concern recognized worldwide (WHO, 2017; ECDC, 2020). AMR bacteria could spread among humans, animals, and the environment, and lead to increased human morbidity and mortality (ECDC, 2020). In the last decade the emergence of Carbapenems-resistant strains is increasing throughout the world, and has been associated to a risk-factor for nosocomial infections, due to this situation they were included in the list of priority pathogens for which Research & Development of new antibiotics are extremely needed, including carbapenem-resistant *Enterobacterales, Pseudomonas aeruginosa* and *Acinetobacter* strains (WHO, 2017). This strains have often been associated with the occurrence of Multidrug resistant (MDR) clones, which is a significant threat due to the few antimicrobial agents that are still effectiveness (Poirel *et al.*, 2011). According to the European Center for Disease Prevention and Control (ECDC), in 2020, the southern and eastern Europe reached the highest percentages of AMR, suggesting and strengthening the importance of the early detection and identification of those strains (Nordmann and Poirel, 2019; ECDC, 2022).

Carbapenemases are enzymes that hydrolyze carbapenems compounds and other β-lactams and is recognized as the most common mechanisms of resistance to carbapenems (Nordmann and Poirel, 2019; Dewi *et al*., 2020). These enzymes has been associated to an easy spread into the community and the environment mainly accomplished through mobile genetic elements, such as plasmids or transposons, which could lead to the horizontal gene transfer among pathogens and might favour the dissemination of AMR to another bacterial niches (Nordmann *et al.*, 2011; Mairi *et al.*, 2018; Chen *et al.*, 2020; Nordmann and Poirel, 2019; Dewi *et al*., 2020; Bonardi and Pitino, 2019). However, other mechanisms of resistance to carbapenems may be reported, such as the loss of outer membrane porins (*e.g.*: *Enterobacterales* carrying Extended-spectrum beta-lactamases (ESBL) or AmpC enzymes), the loss of expression of the OprD porins in *P. aeruginosa*, efflux pumps or even due to mutations in penicillin-binding proteins (*e.g.*: *Escherichia coli*, *P. aeruginosa* and *Acinetobacter baumannii*) (Nordmann and Poirel, 2019; Dewi *et al*., 2020; Public Health England, 2020). Nowadays, some of the most widespread genes of cabapenemases in NFB include Ambler class A: *Klebsiella pneumoniae* carbapenemase (KPC) and Guiana extended-spectrum β-lactamases (GES); class B: Verona integron-mediated metallo-β-lactamases (VIM), Active-on-imipenem (IMP) and New Delhi metallo-β-lactamases (NDM) and class D: oxacillinases (OXA-48) (Bogaerts *et al.*, 2013; Tacäo *et al*., 2015; Bachiri *et al*., 2018; Poirel *et al*., 2011; Nordmann *et al*., 2011; Tiwari *et al.*, 2012; Bonardi and Pitino, 2019, Public Health England, 2020; Lee and Suh, 2021).

The “selective pressure” increase on AMR bacteria had as a starting point, the misuse or overuse of antimicrobials in humans and animals, but may also have been promoted due the presence of heavy

metals in the environment (Rebelo *et al*., 2021). In fact, various studies reported that metal tolerance could participate on the selection of MDR bacteria (Silveira *et al*., 2014; Figueiredo *et al*., 2019) through indirect selection, due to the presence of the genes encoding for both metal and antibiotic resistance located in the same mobile genetic elements such as plasmids (McArthur and Tuckfield, 2000; Romero *et al*., 2017; Figueiredo *et al*., 2019). The heavy metals have a natural ubiquitous distribution in the marine environment. Nevertheless, it is the anthropogenic activity the main source (metal food additives, farming management of biosecurity and hygiene, wastewater, industry and mining) (Rebelo *et al*., 2021; Zagui *et al.*, 2021; Romero *et al.*, 2017; WHO, 2001). Several studies state that even if the marine mammals are not treated with antimicrobials they might be a potential reservoir of resistant bacterial strains that could infect or colonized other animals or humans (Duff *et al.*, 2016; Marti *et al*., 2018; Duff *et al*., 2020; Wallace *et al*., 2013; Frazzon, 2016; Norman *et al.,* 2021). In fact, MA may have the ability to accumulate heavy metals, that could play a role on promoting the capture of AMR genes and will became a reservoir of MDR bacteria, including carbapenemases producing (CP), jeopardizing marine water safety and quality and therefore Public Health (Bonardi and Pitino, 2019; Wallace *et al.*, 2013, Romero *et al*. 2017; Rebelo *et al*., 2021). It is reinforced that MDR bacteria, namely CP, has been scarcely reported in MA, which can be considered as sentinels of marine health (Norman *et al.,* 2021).

The aim of this study is to bring new insights to the status of CP in the MA from Portuguese coast, to assess the anthropogenic activity on their potential dissemination in marine ecosystem.

MATERIALS AND METHODS

STUDY DESIGN

During May and April 2022, a total of 28 samples were collected from viscera of wild fishes, provided from the Atlantic Ocean along the Portuguese Centre coast. The sampling included: Two viscera from *Trachurus trachurus*, one from *Dicentrarchus labrax*, one from *Trisopterus luscus*, eight from *Zeus faber*, six from *Diplodus sargus*, four from *Merluccius merluccius*, three from *Todarodes sagittatus*, two from *Scyliorhinus canicula* and one from *Raja brachyura* (Cohen *et al.*, 1990; Roper *et al.*, 2010; Ebert *et al.,* 2013; Martins, 2018). The transport of the samples was carried out in a refrigerated environment, and all of them were processed immediately upon arrival at the microbiology laboratory: gutting followed by enriched in buffered peptone water, followed by a homogenization in a *stomacher* (“BagMixer”, Interscience®) for approximately one minute and left incubated at 35 ± 2°C overnight.

BACTERIAL SELECTION AND GROW CONDITIONS

For the selection of the carbapenemase-producing *Enterobacterales* (CPE), the samples were plated into the chromogenic culture media ChromID Carba Smart Agar Plates (Biomerieux®), which the CARB compartment medium is selective for the growth of mainly *K. pneumoniae* carbapenemase (KPC) and metallo-carbapenemase-type CPE and the OXA compartment medium for OXA-48-type CPE. The plates were incubated during 18-24 hours at 35 ± 2°C. The selection of the isolates was chosen by phenotypic characteristics. According to the manufacturer's instructions, tested for human clinical specimens, the colonies with a spontaneous coloration (pink to burgundy) could be *Escherichia coli* and the colonies with a spontaneous bluish-green to bluish-grey or purple coloration could be *Klebsiella* spp., *Enterobacter* spp.; *Serratia* spp. or *Citrobacter* spp. (Woodford *et al.*, 2018). The recovered isolates were selected and cultured individually in MacConkey agar plates (Oxoid®), then were cultured on Trypticase soy agar (Liofilchem®) to obtain pure culture.

ISOLATE'S IDENTIFICATION

The amplification and sequencing of 16S rRNA gene was used for identification of representative bacterial isolates as previously described (Héritier *et al*., 2003). The sequencing was performed by Eurofins (https://www.eurofins.pt/) and analyzed using the NCBI Reference Sequence Database - Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov).

STUDY OF ANTIMICROBIAL SUSCEPTIBILITY

The antimicrobial susceptibility testing was performed by the standard disk diffusion procedure on Mueller Hinton agar (Biolab®) as described in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2022) using the following nine antibiotics: piperacillin (PRL, 30µg), piperacillin-tazobactam (TZP, 30-6µg), ticarcillin (TIC, 75µg), ceftazidime (CAZ, 10µg), imipenem (IMP, 10µg), meropenem (MEM, 10µg), ciprofloxacin (CIP, 5µg), amikacin (AK, 30µg) and tobramycin (TOB, 10µg) (Oxoid®).

MDR bacteria was considered when the isolates expressed resistance at least to three or more antimicrobial agents of different categories (Magiorakos *et Al.*, 2012).

STUDY OF CARBAPENEMASES AND HEAVY METAL SUSCEPTIBILITY

Representative isolates carriers of resistance to the carbapenems and suspicious of harboring carbapenemase-encoding genes of both Ambler classes A and B have been screened by PCR: *bla*$_{GES}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{KPC}$, as previously described (Bogaerts *et al.*, 2013).

Genes associated to heavy metal bacterial tolerance were selected according to their potential presence in MA and searched by PCR: copper (*pcoA* - periplasmatic multicopper oxidase; *pcoD* - copper inner membrane pump); silver (*silA* - efflux pump; *silE* - periplasmic silver-sequestration protein); arsenic (*arsB* - efflux pump) and mercury (*merA* - mercuric reductase) (Mourão *et al.*, 2015). The primers, and the PCR conditions are reported in Table 1.

STATISTICAL ANALYSIS

The differences of antimicrobials susceptibility profiles have been made applying the Fisher exact test by using the GRAPHPAD Prism® software (version 8.4.2.).

106 **Table 1.** Primers used for identification and research of carbapenemases and heavy metal tolerance
107 genes.

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

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RESULTS

BACTERIAL SELECTION

The characteristics of the colonies that were growing in the Carba Smart Agar Plates®, were not the colonies expected as the ones described in the manufacturer's instructions. Therefore, the majority of the isolates selected were colourless (n=47), some were brownish (n=9), red (n=2) and green/greenish (n=5).

ISOLATE'S IDENTIFICATION

Representative isolates (n=56) were identified by sequencing of 16S rRNA gene and belong to *Pseudomonas* spp. (n=47; colorless colonies) and *Aeromonas* spp (n=9; brownish colonies), the remaining red and greenish colonies were classified as NFB (n=7).

STUDY OF ANTIMICROBIAL SUSCEPTIBILITY

The findings of antimicrobial susceptibility testing revealed that the isolates were totally resistant to all β–lactams (subclasses: penicillins, cephalosporines and imipenem) and floroquinolones (ciprofloxacin): from the 63 isolates obtained from the viscera of wild fishes, 100% (n=63/63) were resistant to TIC, PRL, TZP, CAZ, IMP and CIP; 13% were resistant to TOB (n=8/63: *Aeromonas* spp., *Pseudomonas* spp. and NFB; n=3/2/3, respectively); 27% to MEM (n=17/63: *Pseudomonas* spp., *Aeromonas* spp., n=16/1, respectively) and all of them were susceptible to AK. The isolates with an intermediate susceptibility were classified as resistant. However, the intermediate resistance has been reported in Chart 1.

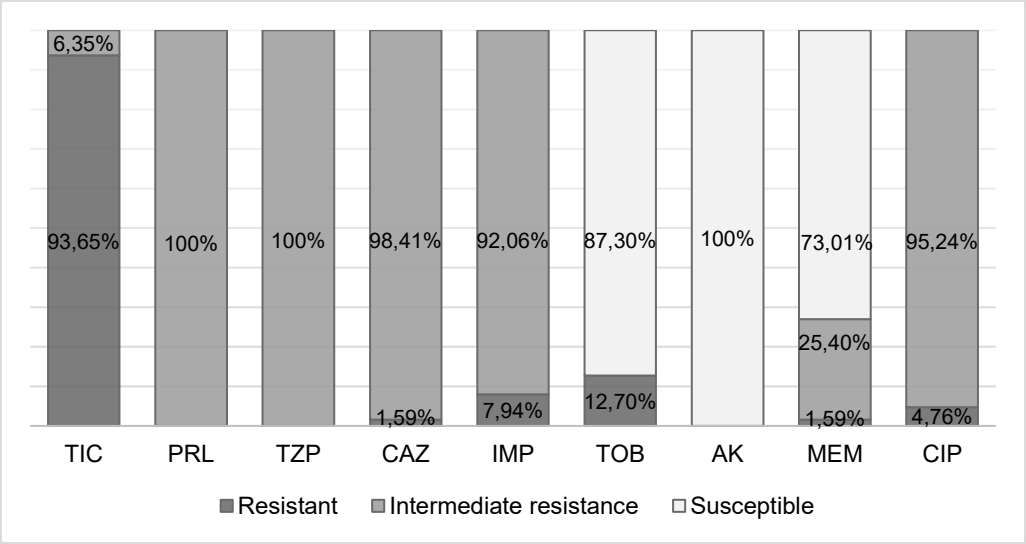

Chart 1. Distribution of antimicrobial resistance among NFB.
P < 0.05 (Fisher's exact test) Ticarcillin (TIC), piperacillin (PRL), piperacillin-tazobactam (TZP), ceftazidime (CAZ), imipenem (IMP), tobramycin (TOB), amikacin (AK), meropenem (MEM), ciprofloxacin (CIP).

The study results revealed seven different antimicrobial resistance profiles (heavy metal tolerance genes were included) (Table 2), noteworthy being the MDR profile "TIC, PRL, TZP, CAZ, IMP and CIP", which represents 61,9% of the isolates (n=39/63: *Pseudomonas* spp., *Aeromonas* spp., and NFB, n=29/6/4, respectively) (chart 2). Among the seven profiles, another three profiles were considered as MDR: one profile include resistance to penicillins, cephalosporins, carbapenems and fluoroquinolones (TIC, PRL, TZP, CAZ, IMP, MEM and CIP; n=16/63*: Pseudomonas* spp., n=16); other to penicillins, cephalosporins, carbapenems, aminoglycosides and fluoroquinolones (TIC, PRL, TZP, CAZ, IMP, TOB and CIP; n=7/63: *Pseudomonas* spp*., Aeromonas* spp*.,* and NFB, n=2/2/3, respectively); finally, the last profile was observed in only one isolate of *Aeromonas* spp., and include resistance to all subclasses of antimicrobials tested (penicillins, cephalosporins, carbapenems, aminoglycosides and fluoroquinolones: TIC, PRL, TZP, CAZ, IMP, MEM,TOB and CIP).

Additionally, it is possible to note three different profiles which included heavy metal tolerance genes (*merA* and *silA*): i) "TIC, PRL, TZP, CAZ, IMP, MEM, CIP and *merA"* which represents 9,5% of the isolates (n=6/63: *Pseudomonas* spp.); ii) "TIC, PRL, TZP, CAZ, IMP, CIP and *merA"* with 6,4% (n=4/63: *Pseudomonas* spp.) and iii) "TIC, PRL, TZP, CAZ, IMP, CIP and *silA"* which depict 4,8% (n=3/63: *Pseudomonas* spp.). Among all isolates only *Pseudomonas* spp. was holder of heavy metal genes.

Table 2. Antimicrobial susceptibility profile.

NFB (n)	Antibiotics	Heavy metal tolerance genes
32	TIC, PRL, TZP, CAZ, IMP, CIP	-
4	TIC, PRL, TZP, CAZ, IMP, CIP	*merA*
3	TIC, PRL, TZP, CAZ, IMP, CIP	*silA*
10	TIC, PRL, TZP, CAZ, IMP, MEM, CIP	-
7	TIC, PRL, TZP, CAZ, IMP, TOB, CIP	-
6	TIC, PRL, TZP, CAZ, IMP, MEM, CIP	*merA*
1	TIC, PRL, TZP, CAZ, IMP, TOB, MEM, CIP	-

Ticarcillin (TIC), piperacillin (PRL), piperacillin-tazobactam (TZP), ceftazidime (CAZ), imipenem (IMP), tobramycin (TOB), meropenem (MEM), ciprofloxacin (CIP).

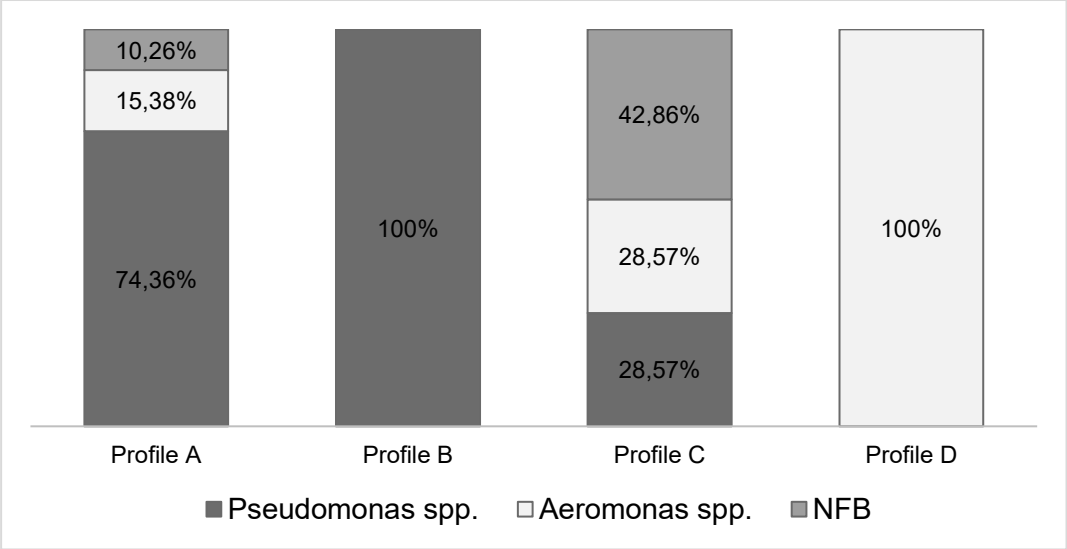

Chart 2. Distribution of the different profiles of AMR, without heavy metal tolerance genes, among different *Genera* or group.

Profile A: TIC, PRL, TZP, CAZ, IMP, CIP (n=39/63); Profile B: TIC, PRL, TZP, CAZ, IMP, MEM, CIP (n=16/63); Profile C: TIC, PRL, TZP, CAZ, IMP, TOB, CIP (n=7/63); Profile D: TIC, PRL, TZP, CAZ, IMP, TOB, MEM, CIP (n=1/63).

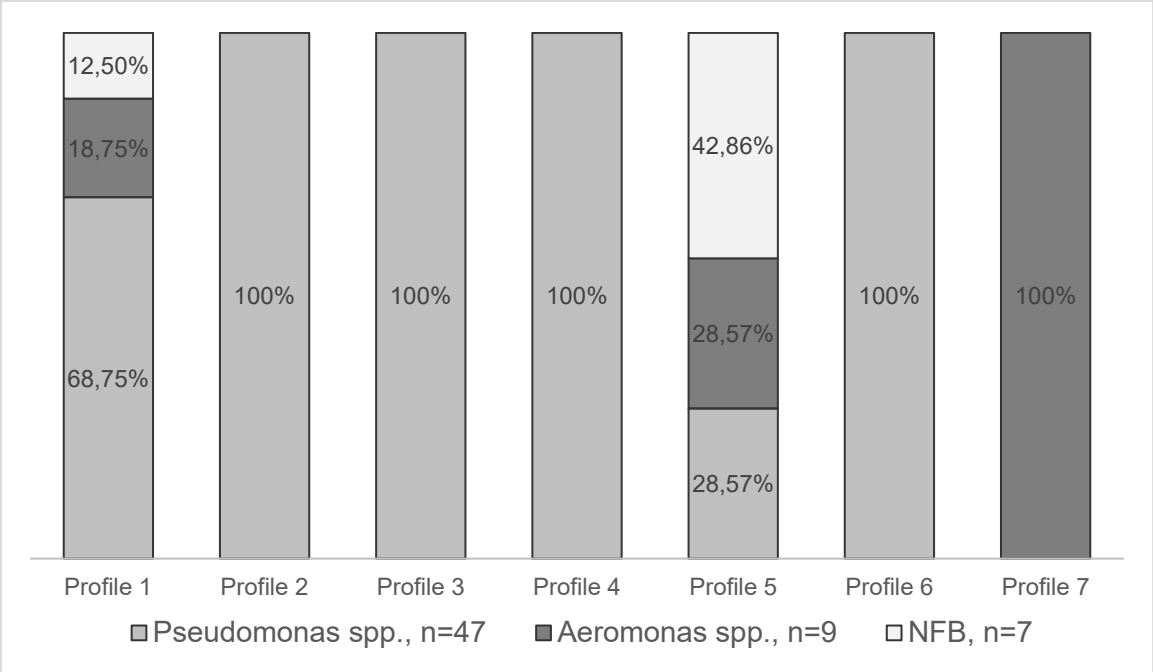

Chart 3. Distribution of the seven different profiles which were included genes codding for heavy metal tolerance.

Profile 1: TIC, PRL, TZP, CAZ, IMP, CIP (n=32/63); Profile 2: TIC, PRL, TZP, CAZ, IMP, CIP, *merA* (n=4/63); Profile 3: TIC, PRL, TZP, CAZ, IMP, CIP, *silA* (n=3/63); Profile 4: TIC, PRL, TZP, CAZ, IMP, MEM, CIP (n=7/63); Profile 5: TIC, PRL, TZP, CAZ, IMP, TOB, CIP (n=10/63); Profile 6: TIC, PRL, TZP, CAZ, IMP, MEM, CIP, *merA* (n=6/63); Profile 7: TIC, PRL, TZP, CAZ, IMP, TOB, MEM, CIP (n=1/63).

STUDY OF CARBAPENEMASE AND HEAVY METAL SUSCEPTIBILITY

After performing a screening of the representative isolates (*Pseudomonas* spp., *Aeromonas* spp. and NFB, n=3/2/1 respectively) for the presence of carbapenemases by PCR, none of the isolates was carrying carbapenemases genes (*bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{IMP}$, *bla*$_{NDM}$ or *bla*$_{VIM}$).

Among the isolates, 15,9% (n=10/63) were carrying the gene *merA* and 4,8% (n=3/63) were presenting the gene *silA.* The *silE*, *pcoA* and *pcoD* and, *arsB* (codding for tolerance to silver, copper and arsenic, respectively) have not been detected. Not having a positive control for the research of *silA*, one isolate has been sequenced and analyzed in the GenBank Blast® (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This sequence had 100% of homology with a conservative protein present in *Pseudomonas* spp. genome with an unknowledge function (WP101321747.1).

DISCUSSION AND CONCLUSIONS

The culture media Carba Smart Agar® is made to select CPE from human clinical specimens. Using samples from fish viscera, the conditions of growth may vary since the bacterial strains may have other individual requirements. Following the manufacturer's instructions, the rise of other MDR bacteria is possible (BioMérieux, 2013), which could explain the growth of *Pseudomonas* spp., *Aeromonas* spp. and NFB instead of *Enterobacterales*. *Pseudomonas* spp. and *Aeromonas* spp. are often found in the gut of carnivore, omnivore as also planktivore fish (Egerton *et al*., 2018) and are ubiquitous in the aquatic environment (Dias *et al*., 2014; Botelho *et al.*, 2019; Haenni *et al*., 2022), which might justify the strong presence of *Pseudomonas* spp. in this study.

The increase of antimicrobial resistance is a world health issue, which acquires even more importance with the emergence of resistances for the last-resort antibiotics such as carbapenems (Tacão *et al*., 2015). The wild marine life, even if not treated by antibiotics, can be an important reservoir of AMRs and could play a significant role for their worldwide propagation, especially with the migratory animals (Marti *et al*., 2018; Chen *et a*l., 2020; Norman *et al*., 2021). Even if some studies have demonstrated the presence of AMR in MA (Norman *et al*., 2021), only a few investigations have been reporting the presence of antimicrobial resistance in wild fish (Arnold *et al*., 2016; Marti *et al*., 2018). The presence of a total resistance to the β-lactams and quinolones tested in this study is due to the fact that a selective medium was used to select carbapenemases, and, consequently, isolates selected can harbored co-resistance to another β-lactams (Nordmann and Poirel, 2019; Dewi *et al*., 2020). The data obtained from this study was that all the isolates were resistant to β-lactams and quinolones tested, some were also resistant to aminoglycosides which could be compared to what is founded in the few published studies, where was described in MA, GNB resistant to β-lactams, aminoglycosides, quinolones and also to cyclines (Blasi *et al.*, 2020; Fernandes *et al*., 2021; Norman *et al*., 2021) and an increase of AMR in the different classes of antimicrobials, which could be compared to the observations made in the terrestrial environment (Wallace *et al*., 2013). In water samples also was reported the presence of similar resistant bacteria (Tacão *et al*., 2015; Montezzi *et al*., 2015; Gambino *et al*., 2022). The presence of AMR in the ocean could come from polluted rivers that flow into the sea and from wastewaters, too (Montezzi *et al*., 2015; Gambino *et al*., 2022).

The heavy metal tolerance genes, *silA* and *merA*, identified in this study, showed that the wild 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). The silver is present naturally in the aquatic environment but at very low concentration (Miller and bruland, 1995). The presence of the gene *silA* could be an indicator to anthropogenic pollution (wastewater discharges) (Tappin *et al*., 2010). To our knowledge, only a few reviews mentioned the *silA* operon and mercuric reductase, as a silver, or mercury tolerance in *Pseudomonas* spp. (Haefeli *et al*., 1984; Wang *et al*., 2022).

As the presence of *silA*, it is natural to find mercury in the ocean at weak concentration, but a higher concentration could come from anthropogenic sources (Zhang *et al*., 2012; Zupo *et al*., 2019). The fish are sensitive to the bioaccumulation of mercury and tend to accumulate higher concentration when they are predatory fish (Evans *et al.*, 2005) which increases the opportunity for heavy metal resistant bacteria. The mechanism of mercury-tolerant bacteria has been investigated because it transforms mercury into a less toxic mercury compound and could contribute to the removal of mercury pollution (Zhang *et al*., 2012). But the genes of antibiotic resistant bacteria and bacteria tolerant to heavy metals are often located on mobile genetic elements such as transposons or plasmids which permit an easier dissemination of the resistances due to horizontal gene transfer (Haefeli *et al*., 1984; Zhang *et al*., 2012; Randall *et al*., 2014; Muller, 2018; Wang *et al*., 2022). In this study, all the isolates carrying heavy metals tolerance genes were identified as *Pseudomonas* spp.. A study already has reported that species like *Aeromonas salmonicida* were having natural exchange of genetic material with *Pseudomonas* spp. and enteric types of plasmids (Botelho *et al*., 2019). The presence of these heavy metal tolerance genes in *Pseudomonas* spp. could be a source for the dissemination to other bacteria, including *Aeromonas* spp. and gut microbiota (Wellington *et al*., 2013). It can also be mentioned that from the 16 isolates with the profile TIC, PRL, TZP, CAZ, IMP, MEM and CIP, 37,5%, (n=6/16) isolates are carrying the gene *merA*, and the difference from the other antibiotics profiles is the resistance to MEM. This could indicate a similar location of the mobile genetic element of the gene *merA* and MEM, among others. Although further research is needed.

Even if carbapenem resistance was mostly found in *Enterobacterales*, it was well disseminated among gram-negative bacteria such as *Pseudomonas* spp. (Dewi *et al.*, 2020) and *Aeromonas* spp. (Stratev and Odeyemi, 2016). In this study, none of the isolates tested was a carrier of carbapenemases genes. Other mechanisms of resistances may be employed by the bacteria, such as the possession of intrinsic resistances to carbapenems due to the production of β-lactamases for the *Aeromonas* spp. or due to the presence of other carbapenemases (*e.g*. OXA-51-like) not tested in this study (Public Health England, 2020). For the *Pseudomonas* spp. other mechanisms of resistance have been described such as the loss of outer membrane porins, the loss of expression of the OprD porins or an overexpression of efflux pumps genes (Nordmann and Poirel, 2019; Botelho *et al*., 2019; Dewi *et al*., 2020). But some studies have already reported the presence of bacteria carrying carbapenemases in MA and aquatic environment (Montezzi *et al*., 2015; Dewi *et al*., 2020). The capacity of the bacteria to transfer the carbapenemases genes due to the mobile genetic element tends to the world propagation (Tooke *et al*., 2019) and may turn the bacteria more virulent (Figueras Salvat and Ashbolt, 2019). In Portugal, a short publication was reported the presence of carbapenemases in bacteria from wild animals, showing the dissemination of those genes in the environment (Dias *et al*., 2014). Some authors advocate about the importance of monitoring these genes in MA, since it can be an indicator of the pollution state of the marine environments (Bonardi and Pitino, 2019). The marine environment could be an underestimate reservoir of carbapenemases, and consecutively a possible via of transmission to human and animals

(Arnold *et al*., 2016): consumption of MA meat, wound infection while manipulating the MA or from other practice activity in marine waters (Wellington *et al*., 2013; Figueras Salvat and Ashbolt, 2019).

In summary, the genus *Pseudomonas* spp., followed by *Aeromonas* spp. are widely present in MA and is a source of AMR, including different MDR profiles. However, in our results they are not a reservoir of carbapenemases. The presence of MDR different profiles in MA poses a risk for the dissemination of resistant strains to humans through water or contaminated fishes. Mores studies are needed to understand the function of the protein coded by the gene *silA* originated from the conservative genome zone of *Pseudomonas* spp.; due to the presence of heavy metal tolerance genes in wild fishes, the presence of co-selection of bacteria resistant to antibiotics is possible. Whereas the contamination of sea environment due to anthropogenic activity could promote the selection of more AMR strains, followed by horizontal transfer through mobile genetic elements between bacteria. Even if these bacteria are not considered pathogenic for humans or animals, once ingested, they can transfer the resistant genes horizontally to the gut microbiota. MA could turn into a reservoir of AMR, becoming a hazard to public health. Further monitoring and systematic surveillance are important to have a better understanding of the status of AMR, including carbapenemases and heavy metal tolerance genes in the aquatic environment bacteria. In fact, this could be considered as an indicator for the pollution level in marine environment.

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APPENDIX

ESCOLA UNIVERSITÁRIA VASCO DA GAMA

mvet MEDICINA VETERINÁRIA

REGISTO DE CASUÍSTICA

Nome aluno (a):	Marine Denise Kameneff												
Local (ais) de estágio :	EUVG	FFUC											
Periodo estágio :	24 / 01 / 2022 a 24 / 05 / 2022												
Breve contextualização do EC:	Foi realizado um projeto de pesquisa e investigação sobre a presença de bactérias produtoras de carbapenemases e portadoras de genes resistentes aos metais pesados encontradas em vísceras de animais marinhos (peixes e moluscos) e que poderiam potencialmente ser uma fonte de transmissão para o homem.												

Animais marinhos	***Trachurus trachurus***	***Dicentrarchus labrax***	***Trisopterus luscus***	***Zeus faber***	***Diplodus sargus***	***Merluccius merluccius***	***Todarodes sagittatus***	***Scyliorhinus canicula***	***Raja brachyura***	**TOTAL**			
Numero de Visceras processadas	2	1	1	8	6	4	3	2	1	28			
TOTAL	2	1	1	8	6	4	3	2	1	28			

Procedimentos e tecnicas realizadas	**Numero de vezes que foi realizado**	*bla*OXA-48-like	*bla*VIM	*bla*IMP	*bla*NDM	*bla*KPC	*pco*A	*pco*D	*mer*A	*ars*B	*sil*A	*sil*E	**TOTAL**
Pesagem das vísceras	28												28
Preeriquecimento das amostras	28												28
Diluições seriadas	28												28
Repicagem de isolados	63												63
Antibiogramas	63												63
Congelação dos isolados	63												63
Extração de DNA	63												63
PCR 16S	63												63
PCR metais pesados	378						63	63	63	63	63	63	378
PCR Carbapenemases	30	6	6	6	6	6							30
Electroforese	408												408
Purificação de produto PCR	63												63
Sequenciação	63												63
TOTAL	1341												1341