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Short Communication

Pseudomonads from wild free-living sea turtles in Príncipe Island, Gulf of Guinea

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ABSTRACT

Dissemination of antibiotic resistance is a major concern, especially in aquatic environments, where pollution contributes for resistant bacteria selection. These strains may have serious health implications, especially for endangered species, including the sea turtles' hawksbill *Eretmochelys imbricata* and green turtles *Chelonia mydas*.

We aimed to evaluate the presence of antibiotic resistant pseudomonads in wild sea turtles from Príncipe Island, São Tomé and Príncipe, Guinea Gulf. Isolates were obtained from oral and cloacal swabs of free-living turtles by conventional techniques. Pseudomonads screening was performed by multiplex-PCR (*oprI/oprL*) and biochemical identification and antibiotic resistance profiling were achieved using Vitek2. All pseudomonad isolates were genotyped by Rep-PCR.

Thirteen isolates were *oprI*-positive and classified as pseudomonads, eight from the genus *Pseudomonas* with the species *P. aeruginosa, P. stutzeri*, and *P. mendocina,* and five co-isolated *Alcaligenes faecalis*. The *P. aeruginosa* isolate was also *oprL*-positive. Regarding isolates susceptibility profile, 38.5% were susceptible to all antibiotics tested, and multidrug resistant (MDR) strains were not identified. DNA fingerprinting did not show any specific clonal-cluster similarity.

Data on the worldwide incidence of antibiotic resistance among wildlife is still very scarce, especially concerning remote tropical areas. Since *Pseudomonas* genus has emerged as a group of increasingly reported opportunistic microorganisms in human and veterinary medicine with high resistance levels, it could be used as a tool for environmental resistance surveillance, particularly considering their ubiquity.

1. Introduction

West coast African countries are important regions as habitats for feeding and nesting of sea turtles, including hawksbills (*Eretmochelys imbricata, Linnaeus*, 1766) and green turtles (*Chelonia mydas, Linnaeus*, 1758) (Formia et al., 2003), two species respectively considered as critically endangered and as endangered, according to The IUCN Red List of Threatened Species (Camacho et al., 2013; González-Garza et al., 2015; Orós et al., 2005). Mortality causes include direct and indirect anthropogenic activities, such as fishing, trauma and pollution, but these animals can also be affected by neoplasia and infectious diseases, namely pneumonia, hepatitis, meningitis and septicemia (Óros et al., 2005).

Although presenting a high longevity, sea turtles are extremely vulnerable to organic and chemical pollution (Foti et al., 2009), which makes them good indicator species for environmental monitoring (Al-Bahry et al., 2009, 2012; Barbour et al., 2007; Foti et al., 2009), namely for evaluating the presence of potentially pathogenic and antibiotic resistant bacteria (Foti et al., 2009). The global dissemination of antibiotic resistance in the environment is a major global concern (Al-Bahry et al., 2009; Foti et al., 2009), especially in aquatic environments, since the presence of sewage, chemicals and other waste products among which antibiotic and pesticide residues may select for resistant bacteria (Al-Bahry et al., 2012; Foti et al., 2009; Kümmerer and Henninger, 2003; Kümmerer, 2004).

Among antibiotic resistant microbiota, special attention has been given to *Pseudomonas* spp., as multi-, extensive- and pan-resistant species and strains are constantly and increasingly emerging (McCarthy, 2015). Pseudomonads, including *Pseudomonas aeruginosa*, are ubiquitous Gram-negative bacteria, present in several ecological

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Table 1
Antibiotic resistance profile of the 13 pseudomonads strains and *Alcaligenes faecalis* obtained from oral and cloacal swabs of free-living turtles of Príncipe Island.

		Antibiotic Class, MIC breakpoint µg/mL												
		Penicillins			Cephalosporins		Monobactams	Carbapenems		Aminoglycosides			Fluoroquinolones	Polymyxin	
Strain	Specie	TIC ≤64 - ≥128	TCC ≤64/2 - ≥128/2	TZP ≤16/4 - ≥128/4	CAZ ≤8 - ≥32	FEP ≤8 - ≥32	ATM ≤8 - ≥32	IPM ≤4 - ≥16	MEM ≤2 - ≥8	AN ≤16 - ≥64	GM ≤4 - ≥16	TM ≤4 - ≥16	CIP ≤1 - ≥4	CS ≤2 - ≥8	Resistance profile
T1-5-O	*P. stutzeri*	<=8 / S	<=8 / S	<=4 / S	<=1 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	-
T1-15-C	*A. faecalis*	>=128 / R	>=128 / R	<=4 / S	4 / S	<=1 / S	>=64 / R	<=0.25 / S	1 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	TIC TCC ATM
T2-6-O	*A. faecalis*	64 / S	64 / S	<=4 / S	2 / S	<=1 / S	32 / R	<=0.25 / S	0.5 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	ATM
T2-10-C	*P. stutzeri*	<=8 / S	<=8 / S	<=4 / S	<=1 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	-
T2-12-C	*P. aeruginosa*	16 / S	16 / S	8 / S	<=1 / S	<=1 / S	2 / S	1 / S	<=0.25 / S	<=2 / S	2 / S	<=1 / S	<=0.25 / S	<=0.5 / S	-
T3-4-O	*P. stutzeri*	<=8 / S	<=8 / S	<=4 / S	<=1 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	-
T3-12-C	*A. faecalis*	32 / S	32 / S	<=4 / S	<=1 / S	<=1 / S	32 / R	0.5 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	ATM
T3-13-C	*P. stutzeri*	>=128 / R	>=128 / R	<=4 / S	4 / S	<=1 / S	>=64 / R	1 / S	1 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	TIC TCC ATM
T5-8-O	*P. mendocina*	>=128 / R	64 / S	<=4 / S	4 / S	<=1 / S	>=64 / R	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	TIC ATM
T7-3-O	*P. stutzeri*	>=128 / R	64 / S	<=4 / S	2 / S	<=1 / S	>=64 / R	1 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	TIC ATM
T9-5-O	*A. faecalis*	64 / S	32 / S	8 / S	4 / S	2 / S	>=64 / R	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	ATM
T9-6-O	*A. faecalis*	32 / S	32 / S	8 / S	4 / S	<=1 / S	>=64 / R	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.5 / S	ATM
T12-6-O	*P. stutzeri*	<=8 / S	<=8 / S	<=4 / S	<=1 / S	<=1 / S	<=1 / S	<=0.25 / S	<=0.25 / S	<=2 / S	<=1 / S	<=1 / S	<=0.25	<=0.5 / S	-

O – oral; **C** – cloacae; Ticarcillin **(TIC),** ticarcillin + clavulanic acid **(TCC),** piperacillin + tazobactam **(TZP),** ceftazidime **(CAZ),** cefepime **(FEP),** aztreonam **(ATM),** imipenem **(IPM),** meropenem **(MEM),** amikacin **(AN),** gentamicin **(GM),** tobramycin **(TM),** ciprofloxacin **(CIP),** and colistin **(CS)**; Resistant values are marked as grey.

environments, such as water, soil and rhizosphere (Pirnay et al., 2002; Pirnay et al., 2005). Besides being recognized as important human opportunistic pathogens responsible for nosocomial infections (Oliver et al., 2015), they are also associated with important animal-related infections, affecting production and companion animals as well as wildlife (Haenni et al., 2015).

The occurrence of antibiotic resistant *Pseudomonas* (Al-Bahry et al., 2009; Barbour et al., 2007; Díaz et al., 2006; Foti et al., 2009) and

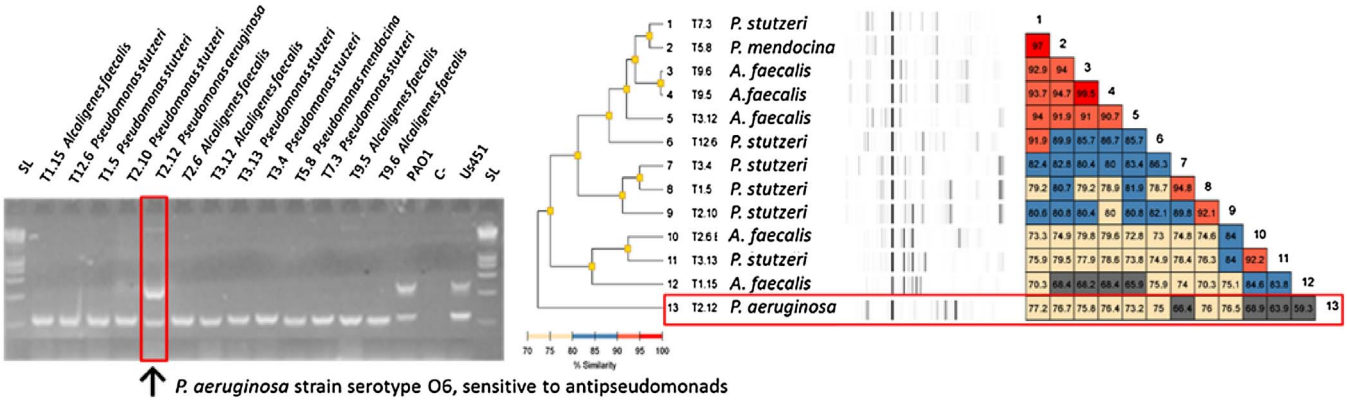

Fig. 1. DNA fingerprinting by Rep-PCR (Diversilab, BioMérieux) of the 13 pseudomonads strains obtained from oral and cloacal swabs of free-living turtles of Príncipe Island.

Pseudomonas-related infections in turtles has been described, especially in pet, farm and zoo turtles, being responsible for shell necrosis, abscess formation, osteomyelitis (Adkesson et al., 2007), pneumonia (Óros et al., 2005), dermatitis (Ladyman et al., 1998; Óros et al., 2005) and conjunctivitis (Di Ianni et al., 2015). Although sea turtles are not considered a relevant source of resistant zoonotic strains, problems may arise due to the increase of eco-tourism and consumption of turtle-derived products, including meat and eggs (Aguirre et al., 2006; Magnino et al., 2009; Warwick et al., 2013).

This work represents a first approach to evaluate the presence of antibiotic resistant pseudomonads in wild free-living sea turtles from Príncipe Island, São Tomé and Príncipe, Gulf of Guinea. Such studies are essential, allowing the establishment of rational environment management and nature conservation programs.

2. Materials and methods

Oral and cloacal AMIES swabs (VWR, Leuven, Belgique) of free-living turtles (*Eretmochemys imbricata*, n = 10 and *Chelonia mydas*, n = 2) were collected in 2010 from animals free of gross signs of disease, in the nearshore waters of Príncipe Island (Gulf of Guinea), during "Programa SADA" (Sustainable Conservation of the Hawksbill Breeding Population at the Príncipe Island, https://tartarugasstomeprincipe.wordpress.com/programa-sada/). Oral and cloacal swab was already described by Dickinson et al. (2001) as a good non-traumatic technique for the characterization of tortoise microbiota and for the assessment of populations' health status. Also, Oliveira et al. (2010) demonstrated that similar collection and transport procedures allow for the isolation of Gram-negative bacteria, even if involving long distances and processing periods.

Sample collected from oral or cloacal were run separately. After sampling, turtles were released back to the sea and swab samples were immediately placed in an icebox, and kept refrigerated until transporting to the Microbiology and Immunology Laboratory from the Veterinary Faculty in Lisbon, Portugal, where were further processed.

Aerobic bacteria isolation from swabs was performed on Columbia agar supplemented with 5% sheep blood (COS, BioMérieux, Marcy-l'Etoile, France), and *Pseudomonas* Selective Agar (CFC, Oxoid, Erembodegem, Belgium), both incubated at 37 °C for 24–48 h. Isolates were characterized through their macro and microscopic morphology, Gram staining characteristics and oxidase reaction. All Gram-negative, oxidase positive bacilli (n = 87) were selected for further characterization.

Pseudomonads screening was performed by multiplex-PCR (*oprI/oprL*), using primers and conditions previously described (De Vos et al., 1997). The *oprI*-positive isolates (n = 13) were then identified biochemically at species level using the Vitek 2 system (BioMerieux, Marcy-l'Etoile, France), and antibiotic resistance (ABR) profiling was performed using the same system in accordance with the manufacturer's instructions. Antibiotic compounds were tested as previously (Serrano et al., 2017) and were as follows: amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, piperacillin + tazobactam, ticarcillin, ticarcillin + clavulanic acid, and tobramycin. Antibiotic resistance phenotypes, represented by the minimum inhibitory concentrations (MICs) were interpreted according to CLSI guidelines for Bacteria Isolated from Animals (CLSI, 2015a). The breakpoints of aztreonam, cefepime, ceftazidime, ciprofloxacin, colistin, meropenem, piperacillin + tazobactam, and tobramycin, not defined at the above CLSI, were interpreted according to CLSI (2015b). *P. aeruginosa* ATCC 27853 was included as control strain.

Additionally, all *Pseudomonas* isolates were genotyped by repetitive extragenic palindromic–polymerase chain reaction (REP-PCR) using the DiversiLabTM system, software version 3.4, (BioMerieux, Brussels, Belgium), while the *P. aeruginosa* isolate was also serotyped (Pirnay et al., 2009).

3. Results

From the 87 oxidase positive isolates obtained, 13 were classified as pseudomonads, based on the presence of *oprI* (14.9%). One of these isolates was also *oprL*-positive, being identified as *Pseudomonas aeruginosa*. According to their biochemical profile, isolates were identified as follows: *Pseudomonas aeruginosa* (n = 1), *Pseudomonas mendocina* (n = 1), *Pseudomonas stutzeri* (n = 6) and *Alcaligenes faecalis* (n = 5).

Regarding strains susceptibility profile, five (total of 38.5%) were susceptible to all antibiotics tested: four *P. sutzeri* and one *P. aeruginosa* (Table 1). Concerning antibiotic resistance (ABR) profile two strains were resistant to ticarcillin, ticarcillin + clavulanic acid, and aztreonam, two were resistant to ticarcillin and aztreonam and four were resistant only to aztreonam. No multidrug resistant (MDR) strains were detected, since none was non-susceptible to at least three antibiotic agents belonging to different categories (Magiorakos et al., 2012).

Genotypic DNA fingerprinting did not show any specific clonal-cluster similarity (Fig. 1). The only *P. aeruginosa* strain identified had a susceptible drug profile and was serotyped as O6.

4. Discussion and conclusion

Sea turtles have been described as potential reservoirs for pathogenic and antibiotic resistant bacteria (Al-Bahry et al., 2009; Warwick et al., 2013), including pseudomonads, which screening is extremely relevant, since they may cause severe infections in these endangered species (Óros et al., 2005 Adkesson et al., 2007; Di Ianni et al., 2015; Ladyman et al., 1998). Therefore, evaluating the presence of antibiotic resistant pseudomonads in wild free-living sea turtles from Príncipe Island is extremely relevant, as this country comprises important areas for turtles' feeding and nesting (Formia et al., 2003). In fact, if

conditions are favorable, these opportunistic bacteria may out-grow the turtles' microbiota and be responsible for increased morbidity and disease dissemination in those wild animal populations (Dickinson et al., 2001), while also contributing for increased environmental shedding (Barbour et al., 2007). They may also have a negative impact on these endangered species reproduction success, as already observed by Stenkat et al. (2014) regarding free-living birds.

Although the percentage of pseudomonads obtained in this study is not high (14.9% – only 13 isolates were classified as pseudomonads between 87 oxidase positive isolates obtained), it should not be neglected. We are aware that it would be desirable to include more samples in this study, but considering the logistics required for sample collection it was not be possible to do so. Isolates were taxonomically allotted to *P. aeruginosa*, *P. stutzeri, P. mendocina,* and *A. faecalis. A. faecalis* are environmental microorganisms that can also be found in the human fecal microbiota, being rarely responsible for human diseases (Chu and Harkness, 2016). Although they can present several resistant traits that can eventually disseminate through the environment to other animals and humans (Zurek and Nayduch, 2016), they were not considered relevant for this study, as are not associated with disease in turtles. Moreover, in De Vos et al. (1997) *Alcaligenes* genus did not tested positive for oprI, contrary to our study. As biochemical identification tests are optimized for human isolates, further molecular studies will be needed to confirm *A. faecalis* identification.

Regarding *Pseudomonas* spp., several species have been already related with disease in turtles (Adkesson et al., 2007; Al-Bahry et al., 2009; Barbour et al., 2007; Díaz et al., 2006; Di Ianni et al., 2015; Foti et al., 2009; Ladyman et al., 1998; Óros et al., 2005) and zoonotic transmission, being associated with several human diseases including otitis, dermatitis, urinary and respiratory infections, especially among cystic fibrosis patients, meningitis, endocarditis and bacteremia (Warwick et al., 2013). The *P. aeruginosa* isolate detected was from serotype O6, a predominant serotype commonly obtained from samples from several origins, including cystic fibrosis patients, human and animal diseases and the environment (Pirnay et al. 2002, 2009). This isolate was included in another study (Serrano et al., 2017) in which it was concluded that animal *P. aeruginosa* population is homogeneously scattered and indistinguishable from the global population structure.

The major concern regarding *Pseudomonas* species is their antibiotic resistance (ABR) ability, as they are intrinsically resistant to several antibiotic classes, but are also able to express acquired resistance traits due to mutations targeting chromosomal genes, efflux pumps, peptidoglycan-recycling genes, porins, topoisomerases and lipopolysaccharides, or due to acquisition of horizontally transferred resistance genes (Oliver et al., 2015). It is also important to refer that these resistant traits can be horizontally transferred to other bacterial species through mobile genetic elements, easily exchanged among phylogenetically distant bacteria, representing an extra concern (Al-Bahry et al., 2009; Farias et al., 2015).

The antibiotic crisis is increasing worldwide (Oliver et al., 2015), including in marine environments, where resistant bacteria can be considered an index of aquatic pollution (Foti et al., 2009). The presence of resistant strains in free-living endangered species of sea turtles is of special concern, due to the probability of transmission to humans, especially local populations, tourists and ecologists working in conservation projects, and other wildlife animals (Barbour et al., 2007).

The fact that none of the *Pseudomonas* strains in this study (and all the *A. faecalis* strains) was considered MDR was expected. The resistance to one or two antibiotic classes observed in eight strains must probably result from intrinsic antibiotic resistance ability of *P. aeruginosa* and its capacity to express acquired resistance mechanisms (Oliver et al., 2015).

Both sea turtles, *E. imbricata* and C. *mydas*, have extensive migratory routes (Camacho et al., 2013; González-Garza et al., 2015; Monzón-Argüello et al., 2011; Orós et al., 2005; Whiting et al., 2008), increasing the probability of contacting with waste products including antibiotic residues, oil spills, heavy metals and pesticides (Al-Bahry et al., 2012; Warwick et al., 2013), which can select for resistant strains (Farias et al., 2015; Foti et al., 2009). Regarding their feeding habits, both species are omnivorous, although preferring sponges and algae (Shuyler et al., 2013; Warwick et al., 2013), which can accumulate chemicals and toxic wastes. Debris ingestion has also been described (Shuyler et al., 2013; Warwick et al., 2013). Also, these turtles can contact other animals that are known reservoirs of resistant bacteria, including pelagic fish (Al-Bahry et al., 2009), wild birds (Stenkat et al., 2014), and sea mammals (Fertl and Fulling, 2007). Therefore, antibiotic resistance can be commonly found in several natural habitats, not necessarily being related with anthropogenic antibiotic administration and application (Farias et al., 2015). Although the presence of ABR *Pseudomonas* in turtles and tortoises was already described by several studies (Al-Bahry et al. 2009, 2012; Barbour et al., 2007; Foti et al., 2009), we did not confirm the presence of MDR pseudomonad strains in this region.

DiversiLab is an efficient method to detect subtle genomic differences when applied to very closely related strains, being more useful for short term studies and outbreaks investigation (Maatallah et al., 2013), whereas sequencing and Multilocus sequence typing (MLST) are more useful for long term epidemiological studies (Spratt, 1999). Although we did not perform sequencing, DiversiLab system results allow concluding that there was none specific clonal-cluster similarity (Fig. 1).

In conclusion, periodical evaluation of the presence of antibiotic-resistant bacteria in endangered turtles can contribute to the success of environmental management programs as previously suggested (Al-Bahry et al., 2009; Barbour et al., 2007). Although MDR strains were not found in wild free-living sea turtles in Príncipe Island, a continuous monitoring of ABR in free-living wild animals should be considered as a tool to help us understand ABR-spread and incidence and find strategies to deal with this worldwide problem. Even in remote locations MDR-bacteria can be found which may represent a risk, especially for the local human populations, considering the limited health assistance and therapeutic options. The whole turtle environment, including migration routes, feeding and nesting areas, should be considered as a potential source and scrutinized in conservation programs.

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