Fecal enterococci from Eurasian otters (*Lutra lutra*) and Neotropical otters (*Lontra longicaudis*): clonality, antimicrobial resistance and virulence traits

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ABSTRACT

The broad use of antimicrobial agents, in both human and animal medicine, and consequently the increase of antibiotic resistant bacteria represents a serious threat to human health worldwide.
Eurasian otter, Neotropical otter and *Enterococcus* spp. isolated from their feces, collected in Brazil and Portugal, in different climatic seasons, were chosen to confirm their potential role as models for assessing the presence of antimicrobial resistance and virulence traits in the environment.
The isolates were identified and characterized at the genus level, species level, and genomic diversity; allowing the selection of a representative isolates collection for each country. Antimicrobial susceptibility and presence of virulence factors was evaluated.
In isolates obtained from scat samples recovered in Brazil, resistance percentage ranged between 14.3% (amoxycillin/clavulanic acid) and 88.6% (clindamycin). In isolates obtained from samples collected in Portugal, resistance percentages ranged between 7.7% (penicillin G) and 84.6% (clindamycin). Significant association was only observed between antimicrobial resistance to individual compounds and country from which the samples were collected, and only for enrofloxacin and vancomycin, whereas for the samples collected in Portugal in different seasons, no significant association was observed between resistance and collection season.
Regarding virulence traits, it was observed that cytolysin and gelatinase were the virulence factors with higher frequency, however, none of the isolates presented DNase and lipolytic activity.
It can be concluded that otters and enterococci belonging to their intestinal microbiota appear to be useful bioindicators for the dissemination evaluation of antimicrobial resistance and virulence factors on aquatic environments.

Key words: *Enterococcus* spp., Eurasian otter, Neotropical otter, antimicrobial resistance, virulence factors.

INTRODUCTION

The increasing dissemination of antimicrobial resistant bacteria, often carrying virulence factors is a global threat to human and veterinary medicines (Holmes et al., 2016; Williams et al., 2016). A possible consequence of this phenomenon is the availability limitation of effective antimicrobials, impairing the treatment of an increasing number of infectious diseases. Besides of the concerns regarding Public Health, there are also economic issues, since these infections sometimes require the use of very expensive next-generation antibiotics (Carroll et al., 2015).
Taking this into consideration, it is necessary to understand how it spreads, how antimicrobial resistance is transmitted and how and why it is maintained within bacterial population.
As the contact between humans and domesticated animals with wild animals is increasing, the potential of dissemination of resistant pathogens to and by wildlife can be considered an emerging problem and should be investigated (Panzenhagen et al., 2016; Usui et al., 2016).
Several studies, already pointed out the raise of antimicrobial resistance on bacteria isolates from wildlife, such as enterococci from the microbiota of otters, that may become environmental reservoirs of drug resistant microorganisms (Oliveira et al., 2010; Oliveira and Ribeiro, 2011; Semedo-Lemsaddek et al., 2013; Prichula et al., 2016).

OTTERS

Otters are semi-aquatic, medium size carnivorous mammals. These animals are in average 60 to 90 cm long and can weight 6 to 10kg. Otters are excellent swimmers, and are capable of ear and see well in and out of the water (Kruuk, 2006).
As an opportunist wild animal, it feeds from week or very small animals; otters`primal food source is mainly fish, but the diet may also include crustaceans, amphibians, birds and small rodents, depending on prey due the availability and habitat (Britton et al., 2017). Generally, each otter has a specific location for scat deposition. River otters are usually active during dusk and down (Kruuk, 2006).
To date, there are 18 otter species identified worldwide, that can be found in a broad variety of aquatic environments, being able to live in salt and freshwaters (IUCN, 2017).
Eurasian otters (Lutra lutra Linnaeus, 1758) can be found in a broad variety of aquatic environments, such as rivers, dams, lakes, marches, streams and coastal areas (Pedroso et al., 2013). From all the species, Eurasian otters have a wider distribution range, being present across Europe and Asia and in some northern Africa countries (Roos et al., 2017).

In 2004, the species classification changed from Vulnerable to Near Threatened on the IUCN Red List, persisting as such until now (Roos et al., 2017). In Portugal, otters present a relatively wide distribution, probably being one of the wider populations of southern Europe (Cabral et al., 2005).
Neotropical otter (Lontra longicaudis, Olfers, 1818) population has the widest geographical distribution among otter species in Latin America, and can be found from Mexico to northern Argentina (Rheingantz and Trinca, 2015). Lontra longicaudis can live on a large variety of territories with distinct characteristics: from warm to cool climates, by the sea or in the rain forest. They have been observed in tropical, humid and deciduous forests, near glacial lakes and up to 4000m altitude from sea level. Their habitat is always associated with the presence of water, such as rivers, lakes, marshes and reservoirs (Rheingantz and Trinca, 2015).
Although otters are susceptible to chemical and organic pollution, they can be found in regions with the presence of human activity like livestock farms and agriculture. This species is now classified as Near Threatened by the IUCN (Rheingantz and Trinca, 2015).
Because of the common features between Eurasian otter and Neotropical otter (both sharing some characteristics, including type of prey, behavior and habitat), they are good models for studies aiming at comparing intestinal microbiota traits.

ENTEROCOCCUS SPP.

Enterococcus spp. belong to the Enterococcaceae family, Lactobacillales order, Bacilli class, Firmicute division and Bacteria kingdom (Devriese et al., 1993). They are gram-positive cells, with spherical or ovoid shape, with size ranging from 0.6-2.0 to 0.6-2.5 µm, that can be displayed as individual cells or arranged in pairs or chains (Thiercelin, 1899). They have reduced flagella, and no capsule or endospores. They multiply usually between 10° C to 45° C, being the optimum temperature of growth 37° C, at pH 9.6 with 6.5% NaCl. Enterococci are oxidase and catalase negative (Devriese et al., 1993).
Their high heat tolerance and ability to survive on hostile environmental conditions allows them to have a wide distribution in nature, and to be great competitors against other bacterial groups (Murray, 1990). They can easily be isolated from soil, water, plants, animals and humans. They are associated with autochthonous microbiota present in the gastrointestinal tract of mammals; rarely cause infection on animals and may act as reservoirs for antimicrobial genes, which can be transferred to animal or human pathogens present in the same habitat (Semedo-Lemsaddek et al., 2003; Williams et al., 2016).

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance is defined as microorganisms inherited or acquired ability, that allows them to resist to the antimicrobial agent's action. When a microorganism is placed in contact with an antimicrobial compound, it is eliminated or it can survive by adaptation (Hollenbeck and Rice, 2012). The coexistence of several mechanisms of resistance to antimicrobials in the same microorganism may lead to a multidrug resistance (MDR) profile, which is defined as a microorganism non-susceptible to at least one agent of three or more antimicrobial categories (Magiorakos et al., 2012).
Resistance may be intrinsic or acquired. Intrinsic resistance occurs without a prior exposure of the bacteria to the antimicrobial compound. Intrinsic resistance mechanisms include cell wall and membrane impermeability to the drug, absence of target receptors for the antibiotic, existence of efflux pumps or antimicrobial compound alteration (Arias and Murray, 2012; Williams et al., 2016). Intrinsic resistance is transmitted vertically to bacteria progeny and is mediated by genes present in the core genome of the species (Williams et al., 2016).
Acquired resistance is the most alarming, because it cannot be predicted and may be transmitted to another bacteria present in the environment. It can be acquired through chromosomal mutations or through horizontally gene transfer (Miller et al., 2014). Mutations can occur spontaneously during DNA replication, giving origin to adaptive mutations. Resistance acquired by horizontal transfer can be achieved through mobile genetic elements such as plasmids (by conjugation or transformation) and transposons (conjugation) (Radhouani et al., 2012).
Intrinsic and acquired resistance traits differ between *Enterococcus* species, the majority carrying intrinsic resistance determinants to β-lactams, low level aminoglycosides, glycopeptides and lincosamides. Concerning acquired resistance, these microorganisms have acquired resistance to glycopeptides, high level aminoglycosides, tetracyclines and quinolones (Cheng et al., 2013; Butler et al., 2014; Kristich et al., 2014; Miller et al., 2014).
A cause frequently related to this increasing problem is the massive use of antimicrobials for the treatment of diseases of both humans and domestic animals. On farms, antibiotics are used to treat diseases, and improve feed efficiency on animals, leading to soil and aquatic contamination. Also, some antimicrobials that were used as growth promoters in production animals are also used in human medicine as observed for amoxicillin and erythromycin (Williams et al., 2016; Vittecoq et al., 2016).

VIRULENCE FACTORS

Antibiotic resistance alone cannot explain the bacteria pathogenic potential, therefore other virulence determinants concerning the pathogenicity of enterococci are also related with this phenomenon (Beceiro et al., 2013).
Virulence factors are substances produced by microorganisms that are responsible for disease development or increased frequency. Additionally,

these might relate to the severity and duration of infections (Beceiro et al., 2013).
These substances are not essential for bacteria survival and are only produced when required. (Carneiro et al., 2015). Regarding *Enterococcus* spp., several virulence factors have been described in the last decade. Gelatinases and cytolysins are two of the most common virulence factor studied. DNase and lipase are also virulence factors described on enterococci, among otters (Barbosa et al., 2010).
Until now most of studies regarding these factors were performed on *Enterococcus faecalis* and *Enterococcus faecium* isolates, and it was observed that they may vary between species. Virulence factors can disseminate between bacteria by gene transfer mechanisms, which may be associated with survival mechanisms. In fact, isolates higher genetic diversity leads to higher survival rates on hosts hostile environments (Manson et al., 2010; Sallem et al., 2014).

MATERIALS AND METHODS

Samples used in this study were collected in two distinct areas. The first area was located along a 13-km stretch, on Paranapanema River, situated in the Campina do Monte Alegre and Paranapanema municipalities, in São Paulo State, Brazil. This river stretch that starts at "Bairro da Ponte", a small fishing village, is located upstream of the Jurimirim hydroelectric station. The evolving area is composed of riverine forests and agro-silvo- pastoral fields (e.g. Eucalyptus, corn and cotton plantations, cattle breeding pastures). The area is characterized by a sub-tropical climate, with temperatures ranged between 22°C in the warmest month (March) and 17°C in the coldest (June).
In Portugal, the study was conducted in Companhia das Lezirias,S.A., an 18,000ha state-owned agro-forestry farmstead located at 40 km northeast of Lisbon (Fig.6). The farmstead has two different geographically separated cores: "Lezíria" (8,000 ha) encompassing rich alluvium soils; and "Charneca" (10,000 ha) characterized by shrubby vegetation and poor, sandy soils. There are 35km of intermittent watercourses carrying water only during high precipitation, 30 small reservoirs, one larger dam and four small streams.

SCAT SAMPLING

Since otters are free-living and mostly nocturnal animals, surveys for both Eurasian and Neotropical otters were performed at the early morning to ensure the freshness of scats sampled, that are easily recognized by their specific characteristics (shape, smell and content), being usually deposited by the otter in prominent places for territory marking. These surveys were conducted by Doctor Nuno Pedroso, supported by grant #2014/08601-6, São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo).
In Brazil, a total of twenty-two (n=22) scat samples were gathered by boat between October and November 2015 along the selected river stretch. Neotropical otter spraints were collected along the river banks, near known otter dens and burrows.
In Portugal, spraint collection (n=28) was performed during foot surveys in two different seasons: September 2015 (n=13) (PT1 - dry season) and February 2016 (n=17) (PT2 - wet season).
For each scat sample selected, an AMIES swab (VWR, Portugal) was immediately performed for bacteria isolation and identification, being kept refrigerated until further processing at the Laboratory of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Lisbon, Portugal.

ENTEROCOCCAL ISOLATION

The isolation of *Enterococcus* spp. from the otters fecal swab samples was performed using specific bacteriological protocols by using Slanetz and Bartley agar medium (SBA) (VWR) supplemented with 0.1% of triphenyltetrazolium chloride (TTC) (VWR), after a 48h at 37ºC incubation. Following incubation, three to four characteristic colonies from each swab were picked randomly and isolated further into pure cultures by streaking on the same medium and incubating for 48h at 37ºC, for 5 consecutive times.

GENUS IDENTIFICATION

All isolates were identified to genus level by gram staining and determination of catalase and oxidase activity, followed by PCR with genus specific primers Ent1 and Ent2 (Ke et al.,1999).
For DNA isolation, isolates were cultured in BHI broth at 37ºC for 16h, followed by DNA extraction using the boiling method (Millar et al., 2000).
For PCR amplification, a mixture was prepared containing 10 µl of Supreme NZYTaq 2x Green Master Mix (NZYTech), 1 µl of primers (0.5 µl of each Ent1 and Ent2 primer) and 8 µl of sterile water, plus 1 µl of DNA, resulting in a total reaction volume of 20 µl. Thermocycler (MyCycler Thermal Cycler, BioRad) conditions were as follows: initial step of 94ºC for 3 min, 35 cycles with 1 min at 94ºC, 1 min at 48ºC, and 1 min at 72ºC, followed by an additional step at 72ºC for 5 min. To the PCR products were added 2 µl of GelRed (iNtRON Biotechnology, Inc, Korea) and were resolved by agarose gel electrophoresis (1.2% w/v) in 0.5X TBE buffer at 110V for 2h and photographed on ImageMaster (Pharmacia Biotech).

ISOLATES GENOMIC TYPING

Genomic typing was performed by PCR fingerprinting, and was achieved using the primers OPC19 (5'-GTTGCCAGCC-3') and (GTG)5 (5´GTGGTGGTGGTGGTG-3´) in independent mixtures containing 10 µl of Supreme NZYTaq 2x Green Master Mix (NZYTech), 1 µl of primer and 8 µl of sterile water, plus 1 µl of DNA. Thermocycler (MyCycler Thermal Cycler, BioRad) conditions were as follows: initial step of 94ºC for 4 min, 40 cycles with 1 min at 94ºC, 2 min at 40ºC and 2 min at 72ºC, followed by an additional step at 72ºC for 10 min. To the PCR products were added 2 µl of GelRed (iNtRON Biotechnology, Inc, Korea) and were resolved by

agarose gel electrophoresis (1.5% w/v) in 0.5X TBE buffer at 120V for 3h and photographed on ImageMaster (Pharmacia Biotech).

SPECIES IDENTIFICATION

To identify the isolates at the species level, a PCR multiplex was performed with the primers FM and FL, selected based on published sequences described by (Jackson et al., 2004).

For each isolate, a mixture was prepared containing 10 µl of Supreme NZYTaq 2x Green Master Mix (Nzytech), 1 µl of each primer (forward and reverse) for the FM gene, 1 µl each primer (forward and reverse) for the FL gene and 7 µl of sterile water. To this mixture 1 µl of the previous extracted DNA was added, resulting in a total reaction volume of 20 µl. Thermocycler (MyCycler Thermal Cycler, BioRad) conditions were as follows: initial step of 95ºC for 5 min, 35 cycles with 1 min at 95ºC, 1 min at 55ºC, and 1 min at 72ºC, followed by an additional step at 72ºC for 10 min. To the PCR products were added 2 µl of GelRed (iNtRON Biotechnology, Inc, Korea) and were resolved by agarose gel electrophoresis (1.2% w/v) in 0.5X TBE buffer at 120V for 1h30 and photographed on ImageMaster (Pharmacia Biotech).

ANTIBIOTIC RESISTANCE PROFILING

Antimicrobial resistance profiling was evaluated by the disk diffusion method, using the methodology and breakpoints determined by the Clinical and Laboratory Standards Institute (CLSI 2013; CLSI 2015). Nine antimicrobial compounds were tested, as follows: amoxycillin/clavulanate (AMC, 30 µg), ampicillin (AMP, 10 µg), chloramphenicol (C, 30 µg), clindamycin (DA, 2 µg), gentamicin (CN, 120 µg), enrofloxacin (ENR, 5 µg), penicillin G (P, 10 units), tetracycline (TE, 30 µg) and vancomycin (VA, 30 µg). All disks were purchased from Oxoid.

STATISTIC ANALYSIS

Fisher´s Exact Test was used to evaluate the association between antimicrobial resistance results and sample origin (country and season). A P-value < 0.05 was considered as a significant association, and a P-value ≥ 0.05 a non-significant association. For statistical analysis purposes, isolates showing intermediate resistance to the antimicrobial compounds tested were considered as resistant.

VIRULENCE FACTORS PROFILING

Production of hemolysin was detected on Columbia agar supplemented with 5% horse blood (bioMerieux). Plates were incubated at 37˚C for 48h under anaerobic conditions, to evaluate the production of β- and α- hemolysins (Pereira et al., 2009).

Gelatinase activity was evaluated on Gelatin Peptone Agar (Liofilchem), incubated at 37ºC for 24h. After incubation, plates were flooded with a saturated solution of ammonium sulfate, which allowed gelatinase-producing isolates to develop transparent halos around the colonies (Semedo et al., 2003).

For detection of lipase activity, enterococci suspensions were inoculated in Tributyrin agar (VWR) incubated at 37ºC for 48h. Lipolytic activity resulted in a transparent halo around the colonies (Cosentino et al.,2004).

For detection of DNase activity, suspensions were inoculated in DNase agar (Liofilchem), incubated at 37ºC for 24h. To evaluate DNase production, the medium was flooded with toluidine blue that results in the development of a bright pink halo around the colonies of the positive isolates (Weckman and Catlin,1957).

The presence of virulence genes cylA and gelE was also determined. Using primers selected based on published sequences described by Eaton and Gasson (2001) and Carlos et al. (2010), a multiplex PCR was performed.

A mixture was prepared containing 10 µl of Supreme NZYTaq 2x Green Master Mix (Nzytech), 1 µl of each primer (0.5 µl forward and 0.5 µl reverse) for the cylA, 1 µl each primer (0.5 µl forward and 0.5 µl reverse) for the gelE and 7 µl of sterile water. To this mixture 1 µl of the previous extracted DNA was added, resulting in a total reaction volume of 20 µl. Thermocycler (MyCycler Thermal Cycler, BioRad) conditions were as follows: initial step of 95ºC for 5 min, 35 cycles with 1 min at 95ºC, 1 min at 55ºC, and 1 min at 72ºC, followed by an additional step at 72ºC for 5 min. To the PCR products were added 2 µl of GelRed (iNtRON Biotechnology, Inc, Korea) and were resolved by agarose gel electrophoresis (1.2% w/v) in 0.5X TBE buffer at 120V for 1h30 and photographed on ImageMaster (Pharmacia Biotech).

RESULTS AND DISCUSSION

ISOLATION AND GENUS IDENTIFICATION

Twenty-two (n=22) fresh scat samples were collected in Brazil; from Portugal, thirteen (n=13) samples were collected in September 2015 (dry season), and seventeen (n=17) samples in February 2016 (wet season).

After purification, presumptive tests were performed followed by a specific PCR protocol (Ent – PCR) in order to confirm the isolates identification as Enterococci, and resulted in resulted in 66 gram positive, catalase and oxidase negative isolates, confirmed by PCR reaction, obtained from scats collected in Brazil; from the samples collected in Portugal, it was possible to confirm 24 isolates collected in the dry season and 24 collected in the wet season.

GENOMIC TYPING

The diversity of all the 114 isolates, previous confirmed as *Enterococcus* spp., were submitted to PCR fingerprinting with primer OPC19 and (GTG)5, followed by hierarchical clustering.

The patterns obtained by PCR fingerprinting were analyzed using the Bionumerics software with the purpose of construct dendrograms for isolates obtained from scats collected in both countries, allowing the selection of representative isolates. To

assess the technique reproducibility, a dendrogram with isolates plus 10% of replicates was constructed.
The dendrograms representing the enterococcal isolates obtained from fecal otter scats from Brazil and Portugal are presented in figure 1 and 2, respectively.
It was possible to confirm a high genomic diversity in the collection, confirming it to be representative of the bacterial diversity occurring in both countries. However, in the dendrogram representing otter enterococci from Brazil is also possible to observe a high genomic similarity between some isolates (BR4B/BR20C) collected on different areas. This could result from the fact that otters may prey along the 13-km stretch of Paranapanema River and leave fecal scats at separated locations (Quadros and Monteiro-Filho, 2001; Kasper et al., 2008).

Regarding the Portuguese collection, the isolates genomic diversity is also high. As expected, same isolates from the same origin and fecal sample (e.g. PT28A, PT28C) with similar patterns were identified as clones; therefore, just one was selected for further analysis. As observed on the dendrogram of the isolates obtained from Brazilian scat samples, there are two isolates, PT33B and PT36A, that presented a similar pattern despite being collected on distinct locations (C01 and TR13).
Thirty-five (n=35) isolates were selected to represent the genomic diversity from enterococci collected in Brazil, fourteen (n=14) to represent isolates collected in Portugal in the dry season, and twelve (n=12) to represent enterococci collected Portugal in the wet season.

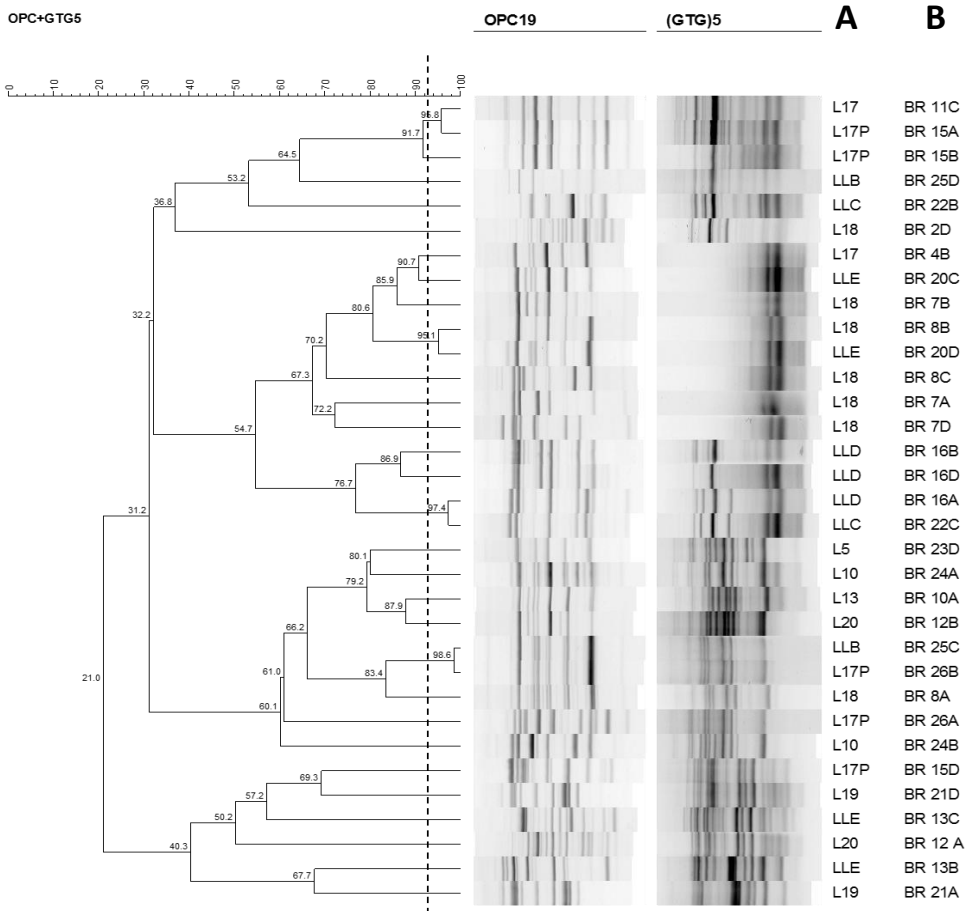

Figure 1- Dendrogram obtained by PCR fingerprinting on the enterococci isolates from otter scat samples collected in Brazil with primers OPC19 and (GTG)5. The first column (A) indicates the sampling site (see Figure 5) and the second column (B) corresponds to the isolate's assigned code. Similarity was calculated with Pearson product-moment correlation coefficient -r- and clustering performed with UPGMA. The dotted-line indicates the reproducibility level (92.7%).

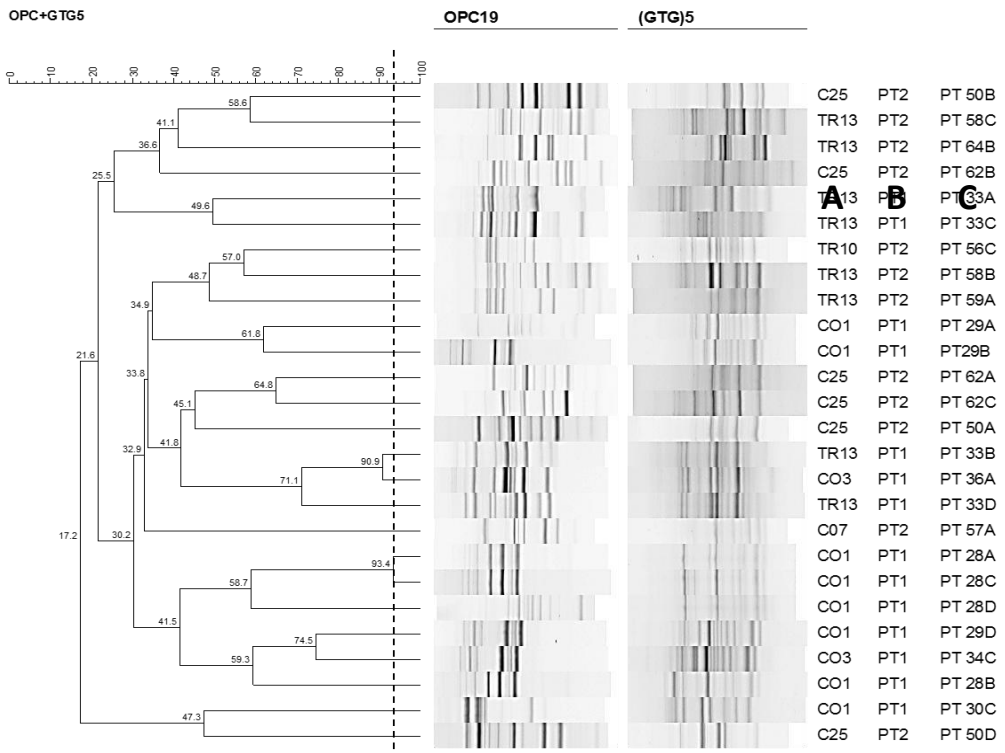

Figure 2- Dendrogram obtained by PCR fingerprinting profiles belonging enterococci isolated from otter scat samples collected in Portugal with primers (GTG)5 and OPC19. The first column (A) indicates the sampling site (see Figure 6), the second column (B) represents the sampling season (PT1-dry, PT2-wet) and the third column (C) corresponds to the isolate's assigned code. Similarity was calculated with Pearson product-moment correlation coefficient -r- and clustering performed with unweighted pair group method with arithmetic mean (UPGMA). The dotted- line indicates the reproducibility level (93.5%).

ANTIMICROBIAL RESISTANCE

The spreading of antimicrobial resistance is considered a risk to public and animal health. In recent years, there has been a growing concern regarding the potential of wild animals in the transmission of antimicrobial resistance bacteria to other animals and even humans (Prichula et al., 2016). The antimicrobial susceptibility test was performed using nine antibiotics compounds frequently used in human and veterinary medicine, and antimicrobial resistant in *Enterococcus* spp. isolates was detected. Brazil samples exhibited the higher resistance level; on the contrary, Portugal isolates presented lower levels of resistance, including the only isolate susceptible to all the antimicrobials tested in this study.

Table 1 and 2 shows the resistance distribution among *Enterococcus* spp. isolates obtained from otter scat samples collected in Brazil (BR) and in Portugal in the dry (PT1) and in the wet (PT2) seasons (respectively). Each table includes the assessment of associations between antimicrobial resistance results and sampling country.

Table 1 - Antibiotic resistance distribution among Enterococcus spp. isolated from otter spraint samples collected in Portugal (PT) and Brazil (BR) and assessment of associations between antimicrobial resistance results and sampling country (Fisher's Exact Test).

	Nº of resistant isolates	Antimicrobial compound									MDR
		AMC	AMP	C	CN	DA	ENR	P	TE	VAN	
PT (n=26)	Total	3	5	7	4	22	14	2	4	13	14
	%	11.5	19.2	26.9	15.4	84.6	53.8	7.7	15.4	50.0	53.8
BR (n=35)	Total	5	9	6	8	31	28	10	12	27	28
	%	14.3	25.7	17.1	22.9	88.6	80.0	28.6	34.3	77.1	80.0
	P=	1	0.7593	0.5284	0.7134	0.5316	0.0492*	0.05467	0.1423	0.03328*	0.0492*

BR – Isolates collected in Brazil; PT – isolates; AMC - amoxycillin/clavulanate, 30 µg; AMP - ampicillin, 10 µg; C - chloramphenicol, 30 µg; DA – clindamycin, 2 µg; CN - gentamicin, 120 µg; ENR - enrofloxacin, 5 µg; P - penicillin G, 10 units; TE - tetracycline, 30 µg; VAN - vancomycin, 30 µg; MDR – multiresistance; P – p-value; *significant associations).

Table 2- Antibiotic resistance distribution among Enterococcus spp. isolated from otter spraint samples collected in Portugal in the dry (PT1) and wet (PT2) seasons and assessment of associations between antimicrobial resistance results and sampling season (Fisher's Exact Test).

	Nº of resistant isolates	Antimicrobial compound									MDR
		AMC	AMP	C	CN	DA	ENR	P	TE	VAN	
PT1 (n=14)	Total	1	2	4	2	12	8	2	2	7	7
	%	7.1	14.3	28.6	14.3	85.7	57.1	14.3	14.3	50.0	50.0
PT2 (n=12)	Total	2	3	3	2	10	6	0	2	6	7
	%	16.7	25.0	25.0	16.7	83.3	50.0	0.0	16.7	50.0	58.3
	P=	0.58	0.635	1	1	1	1	0.4831	1	1	0.7127

PT1 – Isolates from samples collected in Portugal in the dry season; PT2 - isolates from samples collected in Portugal in the wet season); AMC- amoxycillin/clavulanate, 30 µg; AMP - ampicillin, 10 µg; C - chloramphenicol, 30 µg; DA – clindamycin, 2 µg; CN - gentamicin, 120 µg; ENR - enrofloxacin, 5 µg; P - penicillin G, 10 units; TE - tetracycline, 30 µg; VAN - vancomycin, 30 µg; MDR – multiresistance; P – p-value)

Resistance to clindamycin was the most frequently observed, as the resistance levels to this antimicrobial compound were the highest in isolates from samples collected from both countries and Portugal seasons. Since this antimicrobial is applied on humans as an alternative protocol in case of therapeutic failure, the resistance detected may constitute a risk to human health (Pinto et al., 2013). It was also observed a significant association between enrofloxacin and vancomycin resistance and both sampled countries. Enrofloxacin is only used on veterinary medicine and applied on animal production as consequence of its low price (Martins et al., 2015; Panzenhagen et al., 2016). The enrofloxacin resistance observed on the isolates obtained from samples collected in Portugal may result of the intensive use of this antimicrobial compound on the pastures and accumulation on soil near otters habitat. In relation to isolates from Brazil, the results could be explained by the occurrence of direct and indirect discharges on river, containing antimicrobial compounds originated from poultry and cattle farms existing near the areas where the samples were recovered (Fernandes et al., 2015; Williams et al., 2016). In addition, antimicrobial resistance dissemination represents a concern for public health, particularly on the studied area, located in “Bairro da Ponte” near Parapanema river (Brazil), since the river surroundings are inhabited by population which has a close relation with river. They fish on the river and use the water for bathing and domestical activities, leading to an accidentally exposition of the population to these resistant strains.

The recent emergence of vancomycin resistance *Enterococcus* (VRE) strains presents a worldwide problem. Infections caused by vancomycin resistant enterococci are difficult to treat, being associated with high treatment costs, prolonged morbidity, and high mortality rates (Lisboa et al., 2015). Although being usually related to hospital environments, their presence on wild environments is increasing, as determined by several studies (Radhouani et al., 2013; Lozano et al., 2015). A high resistance level to vancomycin was found in the isolates under study, as well as a significant association between vancomycin resistance and isolates origin being observed in isolates collected in both countries and different Portugal seasons. Until now, there are no descriptions available regarding vancomycin resistance in veterinary or environmental bacteria in Brazil. The development of vancomycin resistance is associated with the use of another compound in animal production, avoparcin. Since the use of avoparcin was banned in Europe in 1997, the prevalence of vancomycin resistant bacteria in Portugal is surprising (Ramos et al., 2012).

Regarding the remaining antimicrobials, low levels of resistance to amoxicillin, ampicillin, chloramphenicol, penicillin and tetracycline, were also observed for both countries. Although the isolates collected from samples from Brazil showed a slightly higher antimicrobial resistance to these antimicrobials (amoxicillin: 14.3%, ampicillin: 25.7%, gentamicin: 22.9%, penicillin:28.6%, tetracycline: 35.3%) than isolates obtained from samples collected in Portugal (amoxicillin: 11.5%, ampicillin: 19.2%, gentamicin: 25.4%, penicillin: 7.7%, tetracycline: 15.4%). The antimicrobial resistance to chloramphenicol was the exception, isolates collected from samples from Portugal had higher percentage (26.9%) than isolates obtained from Brazil (17.1%). These data are supported by the fact that *Enterococcus* spp. has intrinsic resistance to several antimicrobial drugs and may acquire resistance mechanisms (Hollenbeck and Rice, 2012; Williams et al., 2016). *Enterococcus* spp. displays several intrinsic resistance mechanisms such as modification of antimicrobial targets, inactivation of therapeutic agents or overexpression of efflux pumps. Several authors have described their intrinsic resistance to β-lactams such as amoxicillin, ampicillin

and penicillin; low level aminoglycosides as gentamicin; and to the phenol class as chloramphenicol. Resistance mechanisms may also derive from the acquisition of mobile elements such as plasmids, transposons or bacteriophages from other *Enterococcus* species or even from bacteria belonging to another genus. It can be also acquired by chromosomal gene mutations. Some studies have already demonstrated acquired resistance on enterococci to tetracyclines and quinolones as enrofloxacin (Cheng et al., 2013; Butler et al., 2014; Kristich et al., 2014; Miller et al., 2014).

The emergence, selection and dissemination of antimicrobial resistant bacteria are caused, in part, by the excessive exposure to antimicrobial drugs (Radhouani et al., 2012). This massive use of antibiotics on human and veterinary medicine to control and treat infection diseases and the use on farms for prevention of infections can also lead to the dissemination through the environment via sewage or discharges. It should be noted that in Europe the use of antimicrobials to enhance animal growth was banned in 2006 (Kemper, 2008), but this ban seems not to have influence the dissemination of resistant bacteria to the environment.

The application of manure or slurry to agricultural areas, the pasture-reared animals excreting directly to land and surface run-off waters may lead to the contamination of aquatic environments (Pinto et al., 2013; Panzenhagen et al., 2016; Usui et al., 2016). The discharge of livestock fecal material on rivers near the sampled area of this study, could be one of the reasons for the existence of antimicrobial resistant enterococci isolates in otter feces. Moreover, these antimicrobial compounds have the propensity to accumulate in the environment, since they are highly absorbed but slowly degraded in soils. The dissemination in aquatic environments and the contact of otters with antimicrobial compounds may turn them into reservoirs of resistant microorganisms (Fernandes et al., 2015; Alexandrino et al., 2016). In the present study, the antimicrobial susceptibility test performed also demonstrated that only one of the isolates was susceptible to all antimicrobials tested, however, a significant percentage of the isolates analyzed demonstrated a multiresistant profile. Eighty percent of isolates obtained from samples collected in Brazil presented it, and on isolates obtained from samples collected in Portugal the percentage was 53.8 % (Table 1). A significant association was also observed between multiresistance and sampled country (Table 1), meaning that resistance profiles vary according to different areas

Regarding isolates collected on separated seasons (wet and dry) in Portugal, the statistical analysis did not show any association between the nine antimicrobials tested and sampling seasons, neither between multiresistance and samples collection seasons (table 2). Previous studies support these results, demonstrating that chemical compounds concentrations on sediments do not vary along the year and with different seasons, being stable near aquatic environments (Fairbairn et al., 2015). In conclusion, resistance variation is mainly associated with sampling areas, and not with seasons.

VIRULENCE FACTORS

One of the major concerns associated with enterococci is related to their ability to express virulence factors, as that can cause damage to the host, contributing to the severity of infections (Hammad et al., 2014). In this study it was also important to evaluate the isolates ability to express virulence determinants as they can be spread through waterways and the environment becoming a risk to humans and animals.

The virulence potential of the otters enterococci under study was first determined by phenotypic methods. Eight isolates originated from samples collected in Brazil were gelatinase-positive (22.9%); 27 isolates produced hemolysin (77.1%) from which 10 isolates produced α-hemolysin (28.6%) and 17 produced β-hemolysin (48,6%); none of the isolates tested produced DNase or lipase (figure 3).

The isolates recovered from samples collected in dry season in Portugal only showed hemolytic activity. Fourteen isolates produced hemolysin (100%).None of the isolates were positive for gelatinase, DNase and lipase production (figure 3).

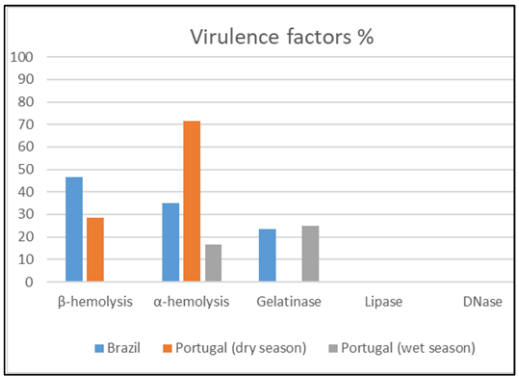

Figure 3- Distribution of virulence factors among *Enterococcus* spp. isolates obtained from otters scat samples collected in Brazil and Portugal

Regarding the isolates collected from scat samples obtained in the wet season in Portugal, 3 were gelatinase-positive (25%); 6 isolates produced hemolysin (50%). None of the isolates were DNase or lipase positive (figure 3).

The lack of ability to produce DNase and lipase by enterococci isolates was already described (Barbosa et al., 2010; Carneiro et al., 2015). Virulence factors are often activated due to environmental pressure. Furthermore, the presence of virulence determinants in commensal isolates may be part of their survival mechanisms that provide better chances to survive in the animal host (Carneiro et al., 2015).

Only the presence of *cyl*A and *gel*E was evaluated by multiplex PCR. It was observed that the *cyl*A gene was present in 6 isolates (17.1%) obtained from samples collected in Brazil and 2 isolates (16.7%) from samples collected in Portugal in the wet season. The presence of *cyl*A was not detected in any

isolate obtained from otter scat samples collected in Portugal in the dry season.

The *gel*E gene was detected in 7 isolates (20%) from samples collected in Brazil and in 3 isolates (25%) from scat samples obtained in Portugal in the wet season. Similar to what was observed for the cylA gene, the presence of gelE was not detected in isolates from samples collected in Portugal in the dry season.

The observation of gelatinase activity and the absence of the virulence factor *gel*E was reported on previous studies (Sallem et al., 2014; Jahan and Holley, 2016) and can be explained by the association of other genes with gelE expression control, like the *fsr* locus. As explained before, gelE operon comprises more genes that can regulate gelatinase activity without the participation of gelE gene. Also, mutated genes can affect *gel*E expression and may regulate gelatinase production (Sallem et al., 2014).

Regarding *cyl*A gene, several isolates exhibited β-hemolytic activity but did not present the *cyl*A gene. This is not unusual since other studies already reported identical results, and can be explained by the fact that more genes control the expression of β-hemolysis, such (Banerjee and Anupurba, 2015).

Overall, a higher frequency of hemolysis and gelatinase activity was observed on isolates obtained from samples collected in both countries. Although not surprising, it can constitute an hazard to humans since hemolysin is a bacterial toxin with major incidence on clinical isolates (Semedo et al., 2003), causing the hemolysis of red blood cells of humans and animal, contributing to the severity of infections (Barbosa et al., 2010).

Gelatinase activity also presents a concern, as it leads to the cleavage of the fibrin layer of the host cells that surrounds the bacteria, allowing the dissemination of the organism in the host, contributing to aggravate tissue injury (Medeiros et al., 2014). The contamination sources of virulent bacteria seem to be the same as for antimicrobial resistant strains, including the sewage and effluent discharged on aquatic environment (Zhang et al., 2016). In this case, the animal farms near sampled areas are the most probable source of bacteria able to produce virulence factors, representing a problem similar to antibiotic resistance. The contact of populations with contaminated water and its use for food and domestical purposes may represent a public health concern, as they can be contaminated by bacterial pathogens harboring virulence traits.

CLOSING REMARKS

Results allowed to conclude that aquatic habitats occupied by otters may act as reservoirs of antimicrobial resistant and virulent bacteria, which may have an impact on population health, especially on inhabitants living near rivers were the samples were collected. It was also observed that resistance profiles are associated with sampling regions and not with sampling seasons, supporting the idea that otters and *Enterococcus* spp. are suitable models to evaluate resistance and virulence dissemination in the wide range of aquatic environments.

The development of strategic management and monitorization plans to reduce environmental contamination by resistant and virulent bacteria and to avoid risks to animal and human health is extremely important, especially in countries where the increasing administration of antimicrobials in veterinary medicine is observed.

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