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Aerobic bacteria found in and around the carcasses of South American Sea Lions, *Otaria flavescens* (Carnivora, Otariidae), in Southern Brazil

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Abstract. Anaerobic bacteria were identified on the body surface and in the beach sand where carcasses of South American sea lion *Otaria flavescens* remain until decomposition. Swabs of the mouth, anus, back and skin of the fins and sand samples around the carcasses were collected from 10 adult specimens, in moderate decomposition state, along the coast of Southern Brazil. The swabs were seeded on blood agar, BHI, MacConkey, Rappaport and Salmonella-Shigella agar and incubated at 37°C for 24-48 hours. Aliquots of 25g of sand were diluted up to 10^3 in 250 mL 0.1% peptone water. A 100 uL volume of each dilution was seeded on BHI agar and MacConkey agar and incubated. The colonies were submitted to Gram stain and identified by using the VITEK® automated system. Thirty-two species of bacteria were isolated and identified. *Proteus*, *Aeromonas*, *Kocuria* and *Sphingomonas* were found in 80% of the examined carcasses. *Proteus vulgaris* and *Sphingomonas paucimobilis* were the most common species in all samples (80%), followed by *Aeromonas salmonicida* (70%), *Kocuria rosea* (60%) and *Streptococcus thoraltensis* (60%). Due to the presence of some bacteria with the potential to infect humans, both in the carcass and in sand nearby, the stranding site should be avoided by people in general.

Key words: pinniped, decomposition, microbial growth, zoonosis.

Resumo: **Bactérias aeróbicas encontradas nas carcaças de leões-marinhos-sul-americanos, *Otaria flavescens* (Carnivora, Otariidae) e sedimento arenoso do entorno, no sul do Brasil.** Bactérias anaeróbias foram identificadas na superfície do corpo e na areia da praia onde carcaças de leão-marinho sul-americano *Otaria flavescens* permanecem até sua decomposição. Amostras da boca, ânus, dorso e pele das nadadeiras e, amostras de areia ao redor das carcaças, foram coletadas em 10 espécimes adultos em estado de decomposição moderado, no litoral sul do Brasil. Os *swabs* foram semeados em ágar-sangue, BHI, MacConkey, Rappaport e ágar Salmonella-Shigella e incubados a 37°C por 24-48 horas. Alíquotas de 25g de areia foram diluídas até 103 em 250 mL de água de peptona a 0,1%. Um volume de 100 uL de cada diluição foi semeado em ágar BHI e ágar MacConkey e incubado. As colônias foram submetidas à coloração de Gram e identificadas pelo sistema automatizado VITEK®. Trinta e duas espécies de bactérias foram isoladas e identificadas. *Proteus*, *Aeromonas*, *Kocuria* e *Sphingomonas* foram encontrados em 80% das carcaças. *Proteus vulgaris* e *Sphingomonas paucimobilis* foram as espécies mais comuns em todas as amostras (80%), seguidas por *Aeromonas salmonicida* (70%), *Kocuria rosea* (60%) e *Streptococcus thoraltensis* (60%). Recomenda-se fortemente que, devido à presença de bactérias com algum potencial zoonótico, o acesso à praia próximo às carcaças de leões-marinhos-do-sul deva ser, de modo geral, evitado pelo público em geral.

Palavras-chave: Pinípede, decomposição, crescimento microbiano, zoonoses.

Introduction

Microorganisms are a significant component of the sand on beaches (Pinto & Oliveira 2011), which is an accumulating element of waste from different origins. This waste acts as a substrate for the presence of parasites and pathogens, which are dispersed in the environment (Lopes *et al.* 2008, Cicero *et al*. 2012). Microorganisms pathogenic to humans and fecal contamination indicators survive for long periods in beach sand due to the presence of organic matter (Vieira *et al*. 2007, Oliveira *et al.* 2015). As reported by the World Health Organization (WHO 1997), bacteria, fungi, parasites, and viruses have been observed in these conditions, and several genera and species are potentially pathogenic to humans through contact.

The proliferation of microorganisms is determined by the availability of organic matter in the sand, which can originate from plant or animal biomass (including decomposing bodies), animal feces and other waste of anthropogenic origin (Vieira *et al*. 2001, Kinzelman *et al*. 2003, WHO 2003). The vulnerability of beaches to pathogenic microorganism development is a public health hazard, because this environment is susceptible to contamination, particularly by helminths and protozoa present in animal feces, including *Ancylostoma* spp. and *Toxocara* spp. (Scaini 2003, Blazius *et al*. 2006, Cáceres *et al*. 2004). However, imbalances caused by this contamination, risks of transmission and even the identification of most pathogenic microorganisms are still unknown. The health and safety of recreational areas are primarily assessed by water quality, but studies have shown that the assessment of the microbiological quality of sand is also relevant (Alm *et al.* 2003, Maier *et al*. 2003, Lopes *et al.* 2008). In this context, sand can directly affect swimmers through causative agents of infectious diseases (Cetesb 2009, Passos *et al*. 2011).

Decaying animal carcasses that remain in coastal areas are an important source of organic matter and pose potential environmental and zoonotic risks. Given the topographical features and limited access to some areas, animal carcasses, such as those from seabirds, marine mammals, fish and even pets are often found along the coastline of Rio Grande do Sul, Brazil (ICMBio 2010). This probably happens because beaches are long and the stranding frequency of pinnipeds and cetacean carcasses is high, especially during the winter months (Pinedo *et al*. 1992). Marine mammals generally have a strong public appeal, as well as scientific and conservation interest. Close contact with the body of these animals, whether by professionals (such as vets or biologists) or by ordinary people out of sheer curiosity, can pose risks to human health, including the transmission of zoonotic diseases and traumatic injuries (Waltzek *et al*. 2012).

Parasitic, fungal, and bacterial diseases have been reported in many marine animals (Dunn 1990, Dunn *et al.* 2001, Silva 1991; Caceres *et al*. 2004; Pereira *et al.* 2020). In Brazil, the presence of *Vibrio* spp. has been identified in various species of marine mammals (Pereira *et al*. 2007), especially cetaceans. Furthermore, in this group of mammals, bacteria of the genera *Staphylococcus, Campylobacter, Aeromonas* and *Vibrio* have been isolated in the Franciscana dolphin *Pontoporia blainvillei* (Pereira *et al*. 2007, 2008, ICMBio 2010). In Sirenia, Delgado (2010) recorded the presence of *Leptospira* spp. in the Amazonian manatee (*Trichechus inunguis*).

In pinnipeds, a wide range of bacteria, including those of public health interest, have been isolated in various geographical areas (Gonzales *et al*. 2009, Carrasco *et al.* 2011). Among isolates is tuberculosis, which has been caused by *Mycobacterium bovis* or *Mycobacterium pinnipedii* in fur seals and sea lions in captivity (Forshaw & Phelps 1991); however, this disease has also been detected in *Arctocephalus australis* and *Otaria flavescens* in the coastal waters of Argentina (O'Reilly & Daborn 1995).

In southern Brazil, *O. flavescens* (Shaw, 1800), also known as the Patagonian sea lion, is endemic. This species is distributed along the rims of both coasts of South America, grouping together on rocky sites. In the Southwestern Atlantic Ocean, the northernmost grouping area of this species is southern Brazil, extending southwards as far as Tierra del Fuego, Argentina and continuing north along the Pacific as far as Peru (Vaz-Ferreira 1981, Rose *et al.* 1994). The species shows significant mortality rates on the southern coast of Rio Grande do Sul State, Brazil, where many animals arrive dead and others die locally, then undergoing decomposition processes (Pavanato *et al.* 2013). These carcasses are not collected and remain for several months under the action of tides, winds, and temperature variations until their total disappearance, by the consumption of scavengers or buried by sand. In the same geographical area, *O. flavescens* has been found dead on the beach and the specimens were highly parasitized by several species of helminths (Pereira *et al*. 2013).

Beach environmental quality has an increasing importance on environmental and public health grounds (Stewart *et al.* 2008) and beach sanitary quality should be monitored not only by the density of fecal coliforms present in waters. Thus, this study aimed to isolate and identify colonies of aerobic bacteria on the body surface of *O. flavescens* in moderate decomposition stage as well as in the soil surrounding the carcass deposition area to check the potential risks to public health.

Material and Methods

Carcass sampling: Swabs from the mouth, anus, back and skin of the fins of corpses of 10 adult specimens of *O. flavescens* found during systematic weekly field trips to the area between Torres (29 ° 20 'S) and Arroio do Chuí (33 ° 15'S), Rio Grande do Sul State, Brazil, were collected from October 2012 to December 2013. The distances between sampling points where the corpses were found did not follow a standard distribution, occurring as close as 300m and as far as 113km apart. Carcasses found in this period were at different stages of decomposition but only those in levels G2 or G3 (G2 – recently dead, fresh cadaver with complete integrity of eyes, and skin with complete fur coat, discrete fermentative process; G3 –relatively fresh cadaver, with complete fur coat, but with initial fermentative process causing abdominal distension, eyes dehydrated) were used, according to the protocol proposed by Geraci and Lounsbury (2005) adapted to pinnipeds. Therefore, the number of sampled sea lions does not represent an estimate of the mortality of this species in the studied area. The swabs were seeded on blood agar, BHI (Brain Heart Infusion), MacConkey, Rappaport and Salmonella-Shigella agar and incubated at 37° C for 24-48 hours.

Sand sampling: Four sand samples (125 $cm^{3)}$ taken from a depth of up to 5 cm and up to 10 cm from the mouth, anus and fins were collected for each carcass. Aliquots with 25g of each sand sample were diluted up to 10^3 in 250 mL 0.1% peptone water. A 100 uL volume of each dilution was seeded on BHI agar and MacConkey agar and incubated at 37° C for 24-48 hours.

After growth, the respective bacterial colonies obtained were submitted to Gram stain and identified by using the VITEK® automated system; bacterial species were considered valid when identity was equal or up to 85%.

The categorization of each bacterial species' zoonotic potential was provided after extensive bibliographical revision, considering as: NF (not found), when no record was found in human species; L (low), with just one record, in a sporadic clinical recording; E (Emerging) previous studies highlighting a new occurrence in more than one given case, considered as "emerging" in the article reviewed; M (medium), with relative occurrence in some geographical areas and in debilitated human patients; H (high), bacteria with recognized zoonotic potential and more than five records in human patients in different geographical areas.

Results

At least 32 species of bacteria from carcass and sand samples (n=80 samples) were isolated and identified (Table I). *Proteus vulgaris* and *Sphingomonas paucimobilis* were the most common species in all samples (80%), followed by *Aeromonas salmonicida* (70%), *Kocuria rosea* (60%) and *Streptococcus thoraltensis* (60%). The percentage of identity of all isolated bacteria is presented in Table I.

Most of all identified bacteria were present in the carcasses, where bacteria of the genera *Aeromonas, Proteus, Kocuria* and *Sphingomonas* were found in 80% of the examined dead animals. Other genera including *Streptococcus* spp. (60%), *Staphylococcus* spp. (50%) and *Acinetobacter* (50%) were also found. *A. salmonicida* was identified in 80% of samples (Figure 1). The presence of *E. coli* was identified in 20% of carcasses and 10% of sand around the 10 carcasses of *O. flavescens*. The genera *Streptococcus* and *Staphylococcus* were isolated in 60% and 50% of the samples, respectively.

When bacterial growth in sand samples was contrasted to that found in carcasses, it was observed that only seven out of 32 identified species were present in both substrates: *A. salmonicida, A. haemolyticus, E. coli, P. vulgaris S. warneri, S. intermedius* and *S. paucimobilis* (Table I, Figure 1).

Discussion

Several microbiological studies have pointed out long lists of species of aerobic bacteria that grow in cultures from samples of animal carcasses on beaches (Gomes 2014). However, the present study has extended the information on sand to monitor potential beach contamination and risks to public health. Even after finding a greater number of bacterial species in decomposing bodies than in the surrounding sand with less than a quarter sharing these resources, it is difficult to guarantee which is the primary source of contamination. These doubts

Table I. Aerobic bacteria isolated from *O. flavescens* carcasses and/or sand around carcasses in southern Brazil and their zoonotic potential**. NF (No record found as zoonosis), L (low), E (emerging), M (medium), H (high).** Bacteria present simultaneously in carcass and sand are typed in bold.

Bacterial Species	% Identities according to VITEK	Sand	Carcasses	Zoonotic potential	References
Aeromonas salmonicida	93	+	+	L,E	Altwegg & Geiss 1989
Aeromonas hydrophila	93	+	-	L,E	
Acinetobacter haemolyticus	90	+	+	L	Almasaudi 2016, Quinteira *et al.* 2007
Acinetobacter ursingii	93	+	-	L	Loubinoux *et al.* 2003
Aloiococcus otitis	99	-	+	L	Pereira *et al.* 2004
Citrobacter freundii	89	-	+	L	Badger *et al.* 1999
Escherichia coli	97	+	+	M	Kosilova *et al.* 2020
Enterococcus faecium	90	-	+	L	Steele *et al.* 2005
Gemela sanguinis	93	-	+	L, E	Purcell *et al.* 2001
Kocuria rosea	94	+	-	L, E	Leclercq *et al.* 1988
Kocuria varians	93	-	+	L, E	Kandi *et al.* 2016
Kocuria kristinae	89	+	-	L	Loong *et al.* 2017
Leuconostoc mesenteroides spp. *cremoris*	93	-	+	NF	Vagiakou-Voudris *et al.* 2002
Pantoea spp.	93	-	+	L	Panditrao & Panditrao 2017
Pediococcus pentosaceus	85	-	+	L	Ludlow *et al.* 2014
Pasteurella pneumotrópica	89	-	+	L	Carriquiriborde *et al.* 2006
Pseudomonas pseudoalcaligens	97	-	+	L	Hage *et al.* 2013
Pseudomonas stutzeri	98	+	-	L	Mattos *et al.* 2013
Proteus vulgaris	99	+	+	L	Burall *et al* 2004
Proteus mirabilis	96	-	+	L	Burall *et al.* (2004
Roseomonas gilardi	99	-	+	L	Malini *et al.* 2017
Rahnella aquatilis	87	+	-	L	Martins *et al.* 2015
Sphingomonas paucimobilis	93	+	+	M	Baddour *et al* 1985
Staphylococcus saprophyticus	86	-	+	L	Rodrigues *et al.* 1993
Staphylococcus lentus	99	+	-	L	Shaker *et al.* 2018
Staphylococcus intermedius	94	+	+	L	Llorca *et al.* 1992
Serratia marcescens	94	+	-	L	Del Peloso *et al.* 2010
Streptococcus thoraltensis	93	-	+	L	Dhotre *et al.* 2016
Stenotrophomonas maltophilia	97	-	+	L,E	Adsul *et al* 2020
Staphylococcus sciuri	88	-	+	L	Hedin and Widerstrom 1998
Staphylococcus warneri	91	+	+	L	Incani *et al* 2010
Shewanella algae	98	+	-	L, E	Bertini *et al* 2018

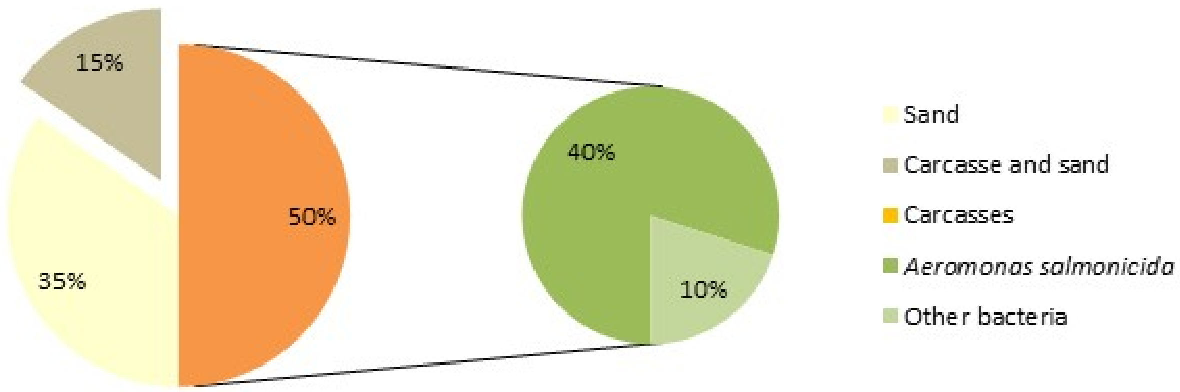

Figure 1. Proportional occurrence (%) of aerobic bacteria species (n=32) in *O. flavescens* carcasses or/and sand around carcasses in southern Brazil. Prevalence (%) is presented to *Aeromonas salmonicida*, considering the 40 samples from carcasses.

may be justified by the presence in the carcasses, even though less significant, of the 16 species which were found in the sand sediment. Considering the beach ecosystem where these carcasses were collected, high interactivity between abiotic and biotic factors in the grounding area was found. The time spent by the carcass on beaches attracts numerous scavengers, including seagulls, vultures, some Falconidae (caracaras), domestic and wild mammals, which may be responsible for the significant presence of organic matter and microorganisms on the site (Gomes 2014). On the other hand, bacteria associated with living sea lions and/or the decomposing tissues of their bodies may undergo washing by the constant action of tides, which, due to salinity, could limit sand colonization by some groups of microorganisms.

Contamination of sand on beaches is a risk to public health, and thus the recognition of each species or group of bacteria present and their potential for infection must always be taken into account. *E. coli* is one of the major constituents of intestinal microbiota in animals and, like thermotolerant coliforms, contaminates sand through feces (Murray 2006). Studies on the occurrence of *E. coli* associated with marine mammals are scarce (Salinas et al. 2010). Infection by *E. coli* is a disease of increasing interest, both to human and animal health, as agents belonging to the family Enterobacteriaceae are responsible for significant incidences of pathological processes in humans and animals (Dho-Molin et al. 1999, Croxen et al. 2010).

Proteus vulgaris is naturally found in the digestive tract of humans and various other animal species (O'Hara et al. 2000). This bacterium is normally found in soil and water contaminated with fecal material and is an opportunistic pathogen known to cause infections in the urinary tract of human beings (Chow et al. 1979), as well as wound contamination (Pearson et al. 2008). Bacteria of the genus Proteus have been identified in the oral cavity of the South American fur seal *Arctocephalus australis* undergoing rehabilitation in southern Brazil (Vargas et al. 2008).

Sphingomonas paucimobilis is another microbial agent typically found in soil, which may occasionally cause septicemia, septic arthritis, and osteomyelitis in humans (Adley & Ryan 2010). Both bacteria were first associated with *O. flavescens* decomposition and may originate and be released during the fermentation process. Given their frequent occurrence, they stand out for their zoonotic potential through contact with mucous membranes and damaged skin, which can represent a gateway for these opportunistic microorganisms.

Aeromonas salmonicida is a common pathogen that causes furunculosis and septicemia in fish (Kamble 2015). This organism usually infects homeothermic vertebrate animals that live in water at low temperatures. Until recently, it was thought that there was no bacterial growth at 37° C; however, a study on the microbiology of marine mammals has identified the presence of several species of *Aeromonas* in cetaceans on the Brazilian coast (Pereira *et al*. 2008). The same authors pointed out the presence of *A. caviae*, *A. hydrophila*, *A. veronii* biogroup sobria, *A. jandaei* and *A. media* in *O. flavescens*. The results of the present study agree with this previous record, because *A. salmonicida* was also the most representative bacterial genus, present in 80% of all samples from the carcasses of *O. flavescens*. The occurrence of *A. salmonicida* reported in this study is new for *O. flavescens* and, even if it cannot be guaranteed as associated with disease in this mammal, Tewari *et al*. (2014) reported the occurrence of this species associated with infection in humans. Species belonging to genus *Aeromonas* have been identified as causative agents of gastroenteritis and skin infections in humans; in addition, they are also considered important fish pathogens (KO *et al*. 2000, Scoglio *et al.* 2001). As *A. salmonicida* was identified both in carcasses and in sand around them, its occurrence seems to be causally linked to the presence of the corpse. The bacterium *A. hydrophila*, despite having been isolated from carcasses of species of cetaceans and even in live *O. flavescens* on the southern coast of Brazil (Pereira *et al.* 2008), was only identified in one sand sample of one animal (10%). This microbe is pathogenic and has been often isolated in samples of various origins, including water and animals, but has been mainly isolated from fish and mussels (Santos 1988, Pereira *et al.* 2004; Harnisz & Tucholski 2010).

Bacteria of the genus *Kocuria* are thought to cause emerging diseases in humans (Savini *et al.* 2010). Although the presence of species of this genus is known to be part of the skin microbiota of man, wild animals, and marine sediment (Kim *et al.* 2004), no records have been found on marine mammals. *Kocuria rosea* was found only in the sand around carcasses in 6 out of 10 animals examined, and its presence could not be associated with carcass decay. Regardless of its origin, this bacterium presents a contamination hazard to humans, since there have been many records of infection in immuno-depressed individuals (Dunn *et al.* 2011).

According to Thornton *et al.* (1998), *Streptococcus* and *Staphylococcus* are commonly found on the skin of marine mammals. *Streptococcus* has subclinical signs, but may develop fever, depression, and various mastitis infections. In debilitated humans, it can cause respiratory symptoms, endocarditis, and skin lesions (Bisno *et al*. 1996). *Staphylococcus* infection in animals can cause skin lesions and pneumonia and eventually evolve to septicemia; in humans, it can cause abdominal pain, vomiting and diarrhea (Devriese *et al*. 2005). Among *Streptococcus*, *S. thoraltensis* is a newly found species that was described by Devriese *et al.* (1997) in cultures originating from the intestine and genital tract of pigs. Although the genus *Streptococcus* now accommodates more than 60 species, relatively few of these streptococcal species have been recovered from marine animals. Indeed, only four species, *Streptococcus phocae* (Skaar *et al.* 1994) and *Streptococcus halichoeri* (Lawson *et al*. 2004) from seals, *Streptococcus iniae* recovered from freshwater dolphins and *Streptococcus marimammalium* from seals (Lawson *et al*. 2005) have been so far identified. In the present study, this species was mostly found in swabs collected directly from the rectum, which excludes the possibility of secondary contamination. This is therefore a new record of *S. thoraltensis* for a marine mammal. Infections in humans are not known, but it is believed that the species usually colonizes the oral cavity (Dhotre *et al*. 2014).

Bacteria with high zoonotic potential, such as *Erysipelothrix insidiosa*, *Leptospira* spp., *Brucella* spp. and *Mycobacterium bovis*, which have already been isolated from pinnipeds (Blank *et al*. 2002), require specific methods for cultivation and identification which were not implemented in this study.

Given the findings of this study, including the presence of some microbial agents with the potential to infect humans, whether in carcasses or sand, access to the beach area next to the animal body may have negative implications for public health, and the use of protective equipment by professionals and researchers responsible for handling them is important.

Conclusions

A great diversity of aerobic bacteria colonizes the body surface of dead sea lions on temperate beaches in Southern Brazil. From the 32 bacterial species identified, most from the sea lion bodies, only seven occurred simultaneously in the carcass and in the sand around it, suggesting low environmental contamination from carcasses. However, it is not yet possible to affirm which the primary source of colonization is, because biotic and abiotic factors interact in the stranding place, given the presence of other vertebrate and invertebrate animals, salinity variations, erosion and dehydration.,

From carcass surfaces, *Proteus*, *Aeromonas*, *Kocuria*, *Sphingomonas*, *Streptococcus*, *Staphylococcus* and *Acinetobacter* were the genera with greatest prevalence. Essentially, considering all samples (body *plus* sand samples = 80 samples) the most frequent bacteria were *A. salmonicida*, *Kocuria rosea* and *Streptococcus thoraltensis*, which accounted for between 60-70% of bacteria found. They do not have a high zoonotic potential except in immunosuppressed individuals, for which sporadic records were found in the literature. As an important recommendation, due to the presence of some bacteria with the potential to infect humans, both in the carcasses of South American sea lions and in sand nearby, the stranding site should be avoided by people in general.

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