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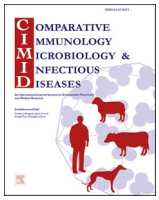

Impact of human created environments in the pathogenic potential and antimicrobial resistance of staphylococci from wild neotropical primates in Brazil

Indiara Sales [a,b], Olney Vieira-da-Motta [b], Ana Tavares [c], Carlos Ramón Ruiz-Miranda [d], Hermínia de Lencastre [c,e], Maria Miragaia [a,*]

[a] Laboratory of Bacterial Evolution and Molecular Epidemiology, Instituto de Tecnologia Química e Biológica António Xavier, NOVA University (ITQB-NOVA), Oeiras, Portugal
[b] Universidade Estadual do Norte Fluminense Darcy Ribeiro-UENF, Centro de Ciências e Tecnologias Agropecuárias, Laboratório de Sanidade Animal-Setor Doenças Infecto-contagiosas, Rio de Janeiro, Brazil
[c] Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica António Xavier, NOVA University (ITQB-NOVA), Oeiras, Portugal
[d] Universidade Estadual do Norte Fluminense Darcy Ribeiro-UENF, Centro de Biociências e Biotecnologia, Laboratório de Ciências Ambientais, Campos dos Goytacazes, Rio de Janeiro, Brazil
[e] Laboratory of Microbiology & Infectious Diseases, The Rockefeller University, New York, USA

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ABSTRACT

The non-human primate (NHP) *Leontopithecus rosalia* is an endangered species native of Brazil and lives in forest fragments with different levels of contact with humans (natural, private and urban). Other NHPs – *Callithrix spp.* - were introduced by humans and co-exist and interact with the native species in these forests.

To evaluate if living in or close to human-modified environments could constitute a risk for *L. rosalia*, we compared the prevalence, genetic background, antibiotic susceptibility and virulence gene content of staphylococci collected from the native and the introduced species from different forest fragments.

We found that presence in human-dominated environments increased the colonization rate of *L. rosalia* with *Mammaliicoccus sciuri* (former *Staphylococcus sciuri*) from 18 % to 85 % ($p = 0.0001$) and of *Callithrix spp* with *Staphylococcus aureus* from 6 % to 100 % ($p = 0.0001$). According to molecular typing data obtained differences probably resulted from dissemination of these bacterial species from the invader NHP species and from humans. Changes in microbiota were paralleled by an increase in the prevalence of Panton-Valentine Leukocidin gene and in resistance to beta-lactams, macrolides and/or lincosamides as exposure to human environment increased. In particular, erythromycin resistance in *S. aureus* from *Callithrix spp*. increased from 0 % to 50 % and resistance rate to at least one antibiotic in coagulase-negative staphylococci species from *L. rosalia* increased from 13 % to 56 % ($p = 0.0003$).

Our results showed that contact of native animal species with human-created environments increased the content of antimicrobial resistant and pathogenic bacteria on their commensal microbiota, which ultimately can impact on their health.

Importance: Endangered animal species are vulnerable to environmental alterations and human activities have been repeatedly identified as factors driving drastic changes in the natural landscape. It is extremely important to monitor changes in the environment surrounding protected species, because this could lead to early detection of any potential threats. In this study, we found that the contact of *L. rosalia* - a protected non-human primate from Brazil - with human environments is related to changes in their commensal microbiota. These included an increase in the number of pathogenic and antibiotic resistant bacteria, which have a higher potential to cause infections that are more difficult to treat. We provided evidence for the harmful impact human contact has on

* Correspondence to: Laboratory of Bacterial Evolution and Molecular Epidemiology, Instituto de Tecnologia Química e Biológica António Xavier, NOVA University (ITQB-NOVA), Avenida da República, Estação Agronómica Nacional, 2780-157 Oeiras, Portugal.
E-mail address: miragaia@itqb.unl.pt (M. Miragaia).

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L. rosalia. Also, our results suggest that monitoring of commensal microbiota of protected animal species might be a useful way of sensing the risks of protected species to human exposure.

1. Introduction

Antibiotic resistance development in bacteria is associated to the continuous use and misuse of antibiotics in humans and human derived activities [1]. Antibiotic resistant bacteria have become wide spread in hospitals, production and companion animals and in human modified environments [2–7]. It has been hypothesized that contact with human and human modified environments and the consequent transmission of potentially pathogenic antibiotic resistant bacteria could constitute a risk to the survival of wild animals, such as the free-ranging neotropical non-human primate (NHP) *Leontopithecus rosalia* (golden lion tamarins).

Staphylococcus aureus, *Mammaliicoccus sciuri* (former *Staphylococcus sciuri*) and other coagulase-negative staphylococci (CoNS) [8,9] are common colonizers of human and non-human primates (NHP) and have been recognized as important reservoirs of antibiotic resistance genes. Moreover, some staphylococcal species, like *S. aureus* have the ability to cause serious infections in NHP, namely septicemia, abscess, stomatitis, arthritis, myocarditis, meningoencephalitis, aerosaculitis, and pneumonia [10–14]. Thus, colonization by antibiotic resistant *S. aureus* might constitute an increased risk to wild NHP as treatment of infections would be more difficult.

Several lines of evidence suggest that transmission of antibiotic resistant bacteria occurs between human and NHP. Examples of this include the finding that old world NHP kept in captivity were colonized by human-related MRSA and MSSA clonal types [14,15]. More scarce, is the information on the colonization of MR-staphylococci in free-ranging neotropical NHP.

Although Brazil is recognized as a country with the largest NHP population worldwide [16], data about their microbiota are scarce [17]. In particular, the genetic characteristics of antibiotic-resistant and pathogenic staphylococci isolated from these animals are poorly characterized, and published information about pathogenicity of *S. aureus* clones in NHP is restricted to captive animals [14].

Golden lion tamarins (*L. rosalia)* are endangered primates endemic to the Atlantic forest of the state of Rio de Janeiro, Brazil. Their population of 2600 animals is distributed over a mosaic of forest fragments with different degrees of protection (biological reserves and private-land forests) that vary in the level of human presence and activity [18,19]. One threat to the conservation of the tamarins is the presence of populations of hybrids of two other Brazilian NHP, the marmosets *Callithrix jacchus* and *C. penicillate* [20]. The introduction of *Callithrix* spp in these forests is a consequence of the illegal wildlife trade [21], therefore this NHP species are managed as invasive species. When in the same forest fragments, marmosets and tamarins interact frequently over food sources [22].

In this study, we aimed to assess if the co-habitation of NHP with humans and human modified environments could constitute a risk for colonization of the native neotropical NHP *L.rosalia* with antibiotic resistant and pathogenic staphylococci. For this purpose, we isolated and compared staphylococci from free-ranging *Callithrix* sp. – the invasive species - and *L.rosalia* – the native species, from areas with different levels of contact with humans: an industrial park, and natural forests with different degrees of human presence.

2. Material and methods

2.2. Study design

Ninety-three non-human primates (NHP) were captured from three regions in Rio de Janeiro, Brazil: an industrial area with a small forest wherein marmosets did not have contact with tamarins, but had extensive contact with humans (Water Island, Ilha d'Água, Guanabara Bay); a rural region wherein marmosets co-exist with tamarins in forest fragments (Private Reserves of National Heritage - RPPNs); and two protected forests in which tamarins do not have contact with marmosets (Biologic Reserve (ReBio) Poço das Antas, Silva Jardim; ReBio União, Rocha Leão) (see Fig. 1).

Tamarins (*L. rosalia*) and marmosets (*Callithrix* spp.) were captured using Tomahawk model traps (18 × 18 × 60 cm) baited with bananas, set on platforms 1.5 m above ground. Traps with captured animals were covered with cloth or paper in order to minimize stress. Tamarins and marmosets from the forest areas were transported by car to the field laboratory at the Mico-leão dourado (*L. rosalia*) Association (AMLD). Marmosets from Water Island were taken to the SERCAS, a captive facility at the Universidade Estadual do Norte Fluminense, where they remained captive. The animals were anesthetized with ketamine hydrochloride (10 mg kg^{-1}) to allow for a clinical examination and the collection of oral and rectal swabs (Cary-Blair, Plast-Labor, Brazil). After recovering from anesthesia, animals were released in the same location they were captured. Although NHP can be colonized in the nose with staphylococci, nasal swabs were not collected. This was due to the fact that besides collecting staphylococci, the project aimed to screen also for other bacteria inhabiting in mouth and rectum/gut. Additionally, staphylococci are known to be also isolated from the mouth and gut.

The tamarins were from groups monitored by the AMLD. For most animals, date of birth and familial relations are known. Among the golden lion tamarins captured, 23 were classified as adults, 17 as young individuals, and seven as infants, with 55.3 % males and 44.7 % females. The marmosets groups had not been part of a long-term monitoring program. The captured marmosets consisted of 19 adults and four young animals, 56.6 % females and 43.4 % males. All animals were apparently healthy when sampled.

2.3. Bacterial isolates

Swabs were enriched in 2.0 mL of BHI broth (HiMedia, India) containing 7.5 % NaCl and aerobically incubated at 37 °C during 24 h. After centrifugation (13.000 rpm, 5 min), pellets were cultured in mannitol salt agar (HiMedia, India) aerobically at 37 °C during 24–48 h and isolates were conserved at − 70 °C. Staphylococci were presumptively identified based on colony morphology, Gram staining, catalase, oxidase, coagulase production (Staphaurex Plus kit, Remel, United Kingdom), and hemolytic pattern (blood agar base supplemented with 5 % (v/v) defibrinated sheep blood aerobically, HiMedia, India).

2.4. Species identification

S. aureus were confirmed by amplification of *nuc* gene by PCR [1] and coagulase-negative staphylococci (CoNS) species were identified by sequencing of an internal fragment of *tuf* gene (elongation factor Tu) [23].

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility was conducted by the agar disk diffusion method (Oxoid, United Kingdom) according to Clinical and Laboratory Standards Institute guidelines [24] for the following 13 antibiotics: penicillin, oxacillin, vancomycin, linezolid, gentamycin, ciprofloxacin, erythromycin, clindamycin, quinupristin/dalfopristin, tetracycline, rifampicin, fusidic acid, and sulfamethoxazole-trimethoprim. For isolates showing resistance or intermediary resistance to oxacillin, vancomycin or fusidic acid, the minimum inhibitory concentration [19] was

determined by Etests (bioMérieux, France), according to CLSI and EUCAST (fusidic acid) [24,25]. The breakpoint for oxacillin in *M. sciuri* was considered to be 3 μg/mL as previously established [26].

2.6. Molecular characterization of S. aureus.

Pulsed-field gele electrophoresis (PFGE) was performed after *Sma*I digestion of total DNA, as described [27]. The resulting band patterns were analyzed with BioNumerics software (version 4.61; Applied Maths, Saint-Martens-Latem, Belgium) using an optimization of 0.5 %, and a tolerance of 1.3 %. Strains were considered to belong to the same PFGE type if the similarity of their macrorestriction was above 80 % and were considered to belong to the same PFGE subtype if their similarity was higher than 95 % [28]. The *spa* type was determined by sequencing as described [29] using the Ridom-Staph software and database (http://spaserver.ridom.de). MLST was conducted [30] for 10 selected *S. aureus* strains, including at least one representative of each *spa* type and PFGE subtype. Gene allele numbers and sequence types (STs) were attributed by using MLST database (http://www.mlst.net/). For the remaining 31 *S. aureus* isolates, the ST was inferred based on the combination of *spa* and PFGE type.

2.7. Detection of virulence factors in S. aureus

ACME allotype (type I to III) [31], and genes codifying the virulence factors Panton-Valentine leukocidin (PVL), LukE LukD leukocidin (*lukED*), class F leukocidin (*lukM*), staphylococcal enterotoxins (*sea-e*, *seg-j*, *sep*, *sel*), toxic shock syndrome toxin (*tsst*), exfoliative toxins (*eta*, *etb*, *etd*), and hemolysins (gamma [hlg], gamma variant [*hlg*v], and beta [*hlb*]) were detected by multiplex PCR, as previously described [32–34].

2.8. Detection of antibiotic resistance genes

The presence of *mecA* and *mecC* genes was determined by uniplex PCR for all *S. aureus* and CoNS isolates [35,36]. Isolates that presented resistance to penicillin but that did not carry *mecA* and *mecC* were additionally screened for *blaZ* by PCR [37] and for *blaZ* and *mecA* by Southern blotting. The *Sma*I DNA macrorestriction fragments were transferred by vacuum blotting [38] to a nylon membrane (GE Healthcare, Amersham, UK) and hybridized with purified PCR amplicons of *mecA* and *blaZ* (1039 bp and 533 bp, respectively) using ECL direct Prime Labeling and detection systems (Amersham Biosciences, Buckinghamshire, UK), according to manufacturer instructions.

Isolates showing resistance to fusidic acid were additionally screened for the presence *fusB* and *fusC* by multiplex PCR, as previously described [39].

2.9. Statistical analysis

The significance of difference between proportions was tested by the Qui2 test using the GraphPad software and considering a 95 % confidence level.

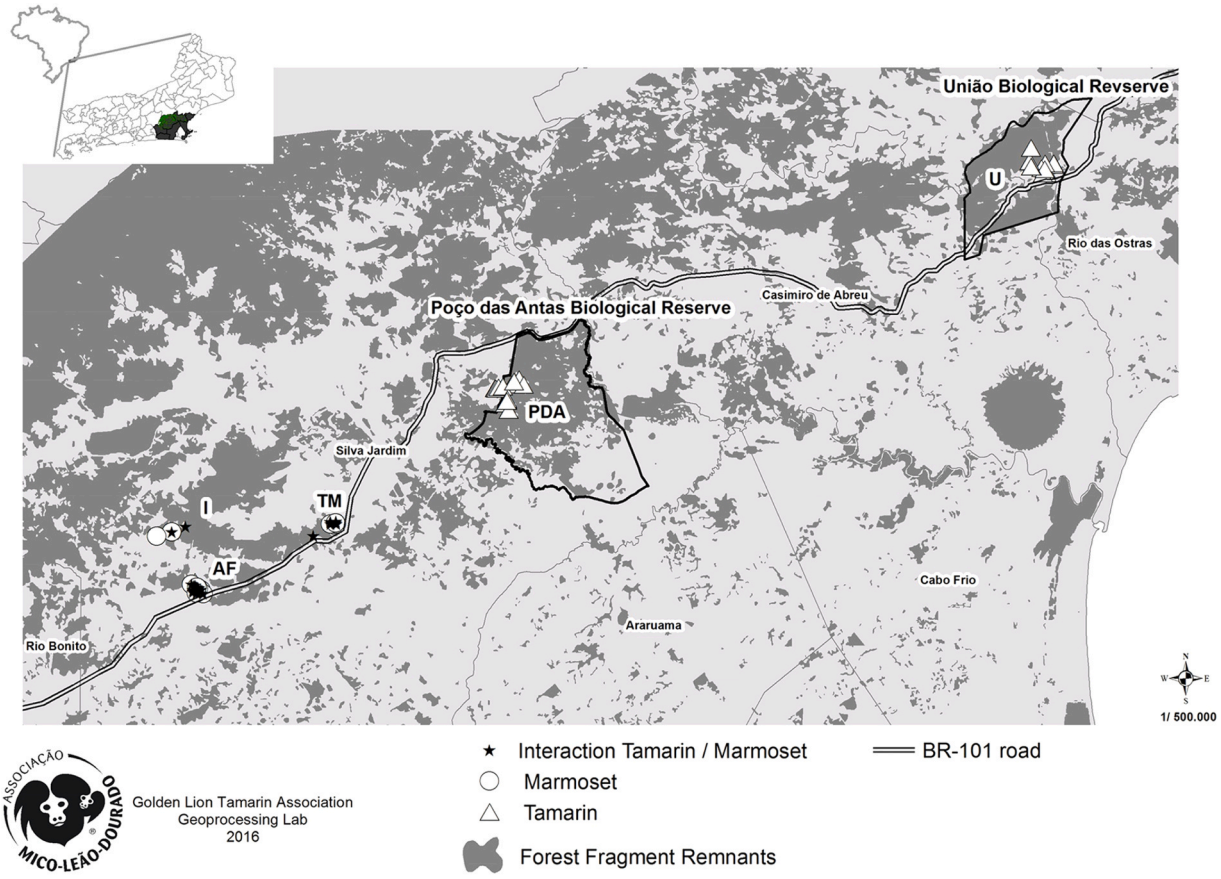

Fig. 1. Map showing the location of the Biological and Private Reservations where *Callithrix spp.* and *L. rosalia* were sampled in this study. Biological reservations (PDA and U) are delimited by a black line; *Callithrix spp.* are represented by a white triangle; *L. rosalia* are represented by white circles; regions of interaction between *L. rosalia* and *Callithrix spp.* are depicted by black stars and constitute the private reservations.

3. Results

3.1. The colonization rate and relative distribution of staphylococcal species among Lion tamarins and marmosets is different

A total of 93 staphylococci (41 *S. aureus* and 52 CoNS) strains were obtained from the 70 NHP analyzed (n = 47 tamarins; n = 23 marmosets); 43 were collected from the rectum and 50 from the oral cavity.

The amount of *S. aureus* colonization in *L. rosalia* (68 %; 32/47) was significantly higher than that of *Callithrix spp*. (39 %; 9/23) (p = 0.03). The overall CoNS colonization rate did not differ between the two species (*L. rosalia*: 76 %; *Callithrix spp*.: 69 %) (p = 0.56). However, they differed in the rate of colonization of individual species: *S. xylosus* colonization rate was higher in *Callithrix spp*. when compared to *L. rosalia* (17 % and 4 %, respectively (p = 0.08) and *S. simiae* colonized *L. rosalia* (21 %) only.

Among all staphylococci, the most frequently isolated species among *L. rosalia* was *S. aureus* (47 %) followed by *M. sciuri* (32 %), *S. simiae* (16 %), *S. xylosus* (3 %) and *S. saprophyticus* (1.5 %). The relative distribution of the staphylococcal species among *Callithrix spp*. was different: *M. sciuri* was the most common species (46 %), followed by *S. aureus*, *S. xylosus* and *S. saprophyticus* (33 %, 17 % and 4 %, respectively) (see Fig. 2) and *S. simiae* was not found.

L. rosalia were colonized by a higher number of staphylococcal species and were frequently co-colonized with different species, a phenomenon that was not observed in *Callithrix spp*. A total of 21 % (10/47) of *L. rosalia* were co-colonized with different pairs of staphylococcal species, including *S. aureus/M. sciuri*; *M. sciuri/S. simiae*, and *S. aureus/S. xylosus*.

There was no difference in distribution of staphylococci between young and adult animals. The data showed a higher prevalence of staphylococci in the oral cavity (71 %, 50/70), compared to rectal cavity (61 %, 43/70) (p = 0.2829), although distribution of individual species among the sites of collection was similarly proportional. The only exception was *S. simiae* that was more frequently isolated from the rectal cavity.

3.2. Contact of non-human primates with anthropogenic environments was associated to a change in the relative prevalence of staphylococcal species

We observed that NHPs living in the urban/industrial environment were colonized by specific staphylococcal species. In privately owned forest 6 % (1/17) of the marmosets were colonized with *S. aureus*, but in the industrial environment (Water Island), all marmosets were colonized with *S. aureus* (p = 0.0001). We also found that *L. rosalia* from private forest fragments were significantly more colonized with *M. sciuri* (18/21, 85 %) than those collected from biological reserves (4/22, 18 %) (p = 0.0001), where the contact with humans is rare or inexistent (see Fig. 3A).

3.3. Staphylococcus aureus strains collected from lion tamarins and marmosets are genetically different

S. aureus isolates recovered from *L. rosalia* were different from *S. aureus* collected from *Callithrix spp*. In *L.rosalia* seven clonal types were found: ST6-t701 (n = 17), ST188-t189 (n = 6), ST6-t5271 (n = 5), ST6-t7396 (n = 1), ST2985-t189 (n = 2) and ST133-NT (n = 1). In *Callithrix spp*., only three clonal types were found: ST398-t1451 (n = 7), ST1-t13736 (n = 1), and ST2984-t13737 (n = 1) (see Table 1 and Fig. 4). From the clonal types identified, the most widespread were the ST6-t701 (n = 30 countries), the ST188-t189 (n = 30) and the ST398-t1451 (n = 15) (SpaRidom database), which were all previously described in human and animal [40–45]. The remaining six clonal types identified in this study were not reported in the literature, but were listed in the SpaRidom database as being isolated in only one or two countries. Noteworthy, only the clonal type ST398-t1451 was previously reported in Brazil [46].

Additionally, the *S. aureus* clonal types identified depended on the geographic origin of the NHP *L. rosalia* analyzed. *S. aureus* collected from *L. rosalia* inhabiting the Biological Reserves belonged mainly to ST6 (81 %; 22/27); and those inhabiting Private Forest Reserves areas were mostly from ST188 (67 %;4/6). For *Callithrix spp.*, the *S. aureus* found in the animals from Water Island belonged mainly to ST398 (87.5 %; 7/8) (Table 1). Interestingly, this was the only staphylococcal species isolated from this specific group of primates. The results suggest that not only each mammalian species has a specific microbiota but also that dissemination of *S. aureus* between the two species of non-human primates is rare. Furthermore contact with human created environment lead to changes in microbiota of both *L. rosalia* and *Callithrix spp.*

3.4. S. aureus collected from non-human primates in anthopogenic environments have decreased susceptibility to erythromycin and carry PVL

Overall, *S. aureus* isolated from non-human primates were highly susceptible to almost all classes of antibiotics tested (see Table 1), which was expected given the low use of antibiotics in this type of natural environments. All isolates were susceptible to penicillin and oxacillin, and carried no *mecA* or *mecC* genes, as determined by PCR and Southern blotting. The only antibiotic for which a decreased susceptibility was observed was erythromycin. A total of 10 % of the strains (4/41), all isolated from *Callithrix spp*. from Water island and belonging to ST398-t1451, were resistant to erythromycin, suggesting that contact with humans might constitute a risk factor for the acquisition of antibiotic

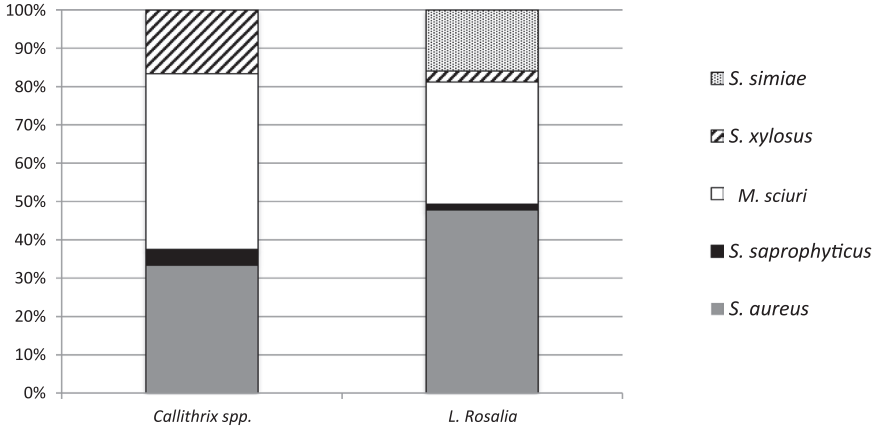

Fig. 2. Distribution of *Staphylococcus* species among *L. rosalia* and *Callithrix spp*.

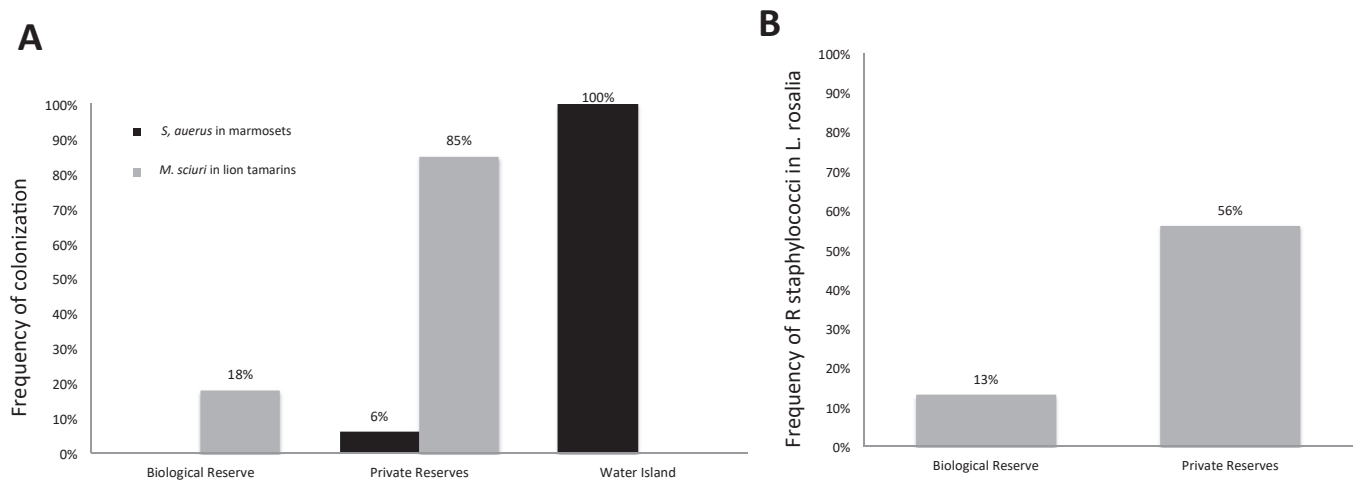

Fig. 3. Frequency of colonization with staphylococci of non-human primates from environments with different levels of contact with humans. A) Frequency of colonization of *Callithrix spp*. with *S. aureus* and of *L. rosalia* with *M. sciuri*; B) Frequency of colonization of *L. rosalia* with staphylococci resistant to at least one antibiotic tested.

Table 1
Genotypic and phenotypic characteristics of *S. aureus* collected from non-human primates in environments with different levels of contact with humans.

Environment	Level of human contact	Host (no isolates)	CClonal type by MLST-*spa* type (MLST-SCC*mec*) (no isolates)	Antibiotic resistance (no isolates)	Virulence genes (no isolates)
Water Island	High	*Callithrix sp.* (8)	ST398-t1451 (7)	Ery^R (4), Fus^R (3)	*hlg* (3)
			ST2984-t13737 (1)	–	*hlgv, lukE/D* (1)
Private Reservations	Low	*L. rosalia* (6)	ST188-t189 (4)	–	*hlb* (4), *hlgv* (4), *lukED* (4), PVL (4)
			ST133-NT (1)	–	*hlb* (1)
			ST6-t701 (1)	–	hlb (1), hlgv (1), lukED (1)
		Callithrix sp. (1)	ST1-t13736 (1)	–	*hlb* (1), *hlgv* (1), *lukED* (1), PVL (1)
Biological Reservations	None	*L. rosalia* (26)	ST6-t701 (16)	–	*hlb*, (16), *hlgv* (16), *lukED* (16) hlb hlgv lukED
			ST6-t5271 (5)	Ery^I (1)	*hlb*, (5), *hlgv* (5), *lukED* (5)
			ST188-t189 (2)	–	*hlb*, (2), *hlgv* (2), *lukED* (2)
			ST2985-t189 (2)	–	*hlb*, (2), *hlgv* (2), *lukED* (2)
			ST6-t7396 (1)	–	*hlb*, (1), *hlgv* (1), *lukED* (1)

resistant bacteria. Strains belonging to this clonal type with resistance to erythromycin were previously described as a cause of infections in humans [42] and an isolate belonging to a related clone (ST398-t034), also resistant to macrolides, was found as a cause of a fatal pneumonia in an oncology patient in São Paulo, Brazil [47].

To understand the pathogenic potential of *S. aureus* isolated from non-human primates *S. aureus* isolates were screened for a set of virulence factors by PCR. The great majority of strains carried LukE-LukD leukocidin (80 %) and hemolysins beta and gamma variant (76 %, each), including representatives of almost all the clonal types (ST1, ST6, ST188, ST2984 and ST2984). The only exceptions were the isolate belonging to ST133 that carried beta-hemolysin only and isolates of ST398 that carried the gamma-hemolysin only (3/7). A total of 12.2 % (5/41) of the isolates carried PVL, including isolates of ST188 collected from *L. rosalia* (n = 4) and isolates of ST1 (n = 1) from *Callithrix spp.*, all collected from private forests. Strains belonging to these genetic backgrounds and carrying PVL have been found to cause infections in humans in different regions of the world, including in Brazil [48].

3.5. CoNS staphylococci can disseminate between non-human primate species

M. sciuri isolates showed a high genetic diversity, comprising as many as 19 different PFGE types (Table 2). Overall, isolates belonging to the same PFGE type were collected from the same species of primates and the same geographic region, suggesting the occurrence of dissemination of isolates between animals that are in close contact. Examples of this are isolates SJ21, SJ22 and SJ23, all belonging to PFGE type F and collected from different animals inhabiting RPPN FC (PT2). However, we also observed the occurrence of the same PFGE in *M. sciuri* isolates collected from *L. rosalia* inhabiting different geographic locations (see Table 2). In addition, we identified one case in which *M. sciuri* isolated from one *L. rosalia* has a *Sma*I macrorestriction pattern (PFGE type E) similar to a *M. sciuri* isolated collected from a *Callithrix spp.* from the same private forest, suggesting that, although rare, dissemination of *M. sciuri* between the two non-human primates might occur. For the remaining CoNS species, we observed a similar situation, wherein related *Sma*I macrorestriction PFGE patterns were found both in isolates sharing the same host and geographic region and in isolates from different geographic places.

3.6. CoNS collected from marmosets in contact with humans are reservoirs of antibiotic resistance

Although in general CoNS were susceptible to most of the antibiotics tested, their frequencies of resistance were higher than those observed for *S. aureus*. *M. sciuri* and *S. xylosus.* were the CoNS species that showed the highest resistance rates and the species that accumulated resistance to more antibiotics. *M. sciuri* isolates showed resistance to penicillin (n = 6/33), oxacillin (n = 2/33) and fusidic acid (n = 33/33) and intermediary resistance to clindamycin (n = 2/33) and *S. xylosus* showed resistance to penicillin (2/6), erythromycin (3/6) and a

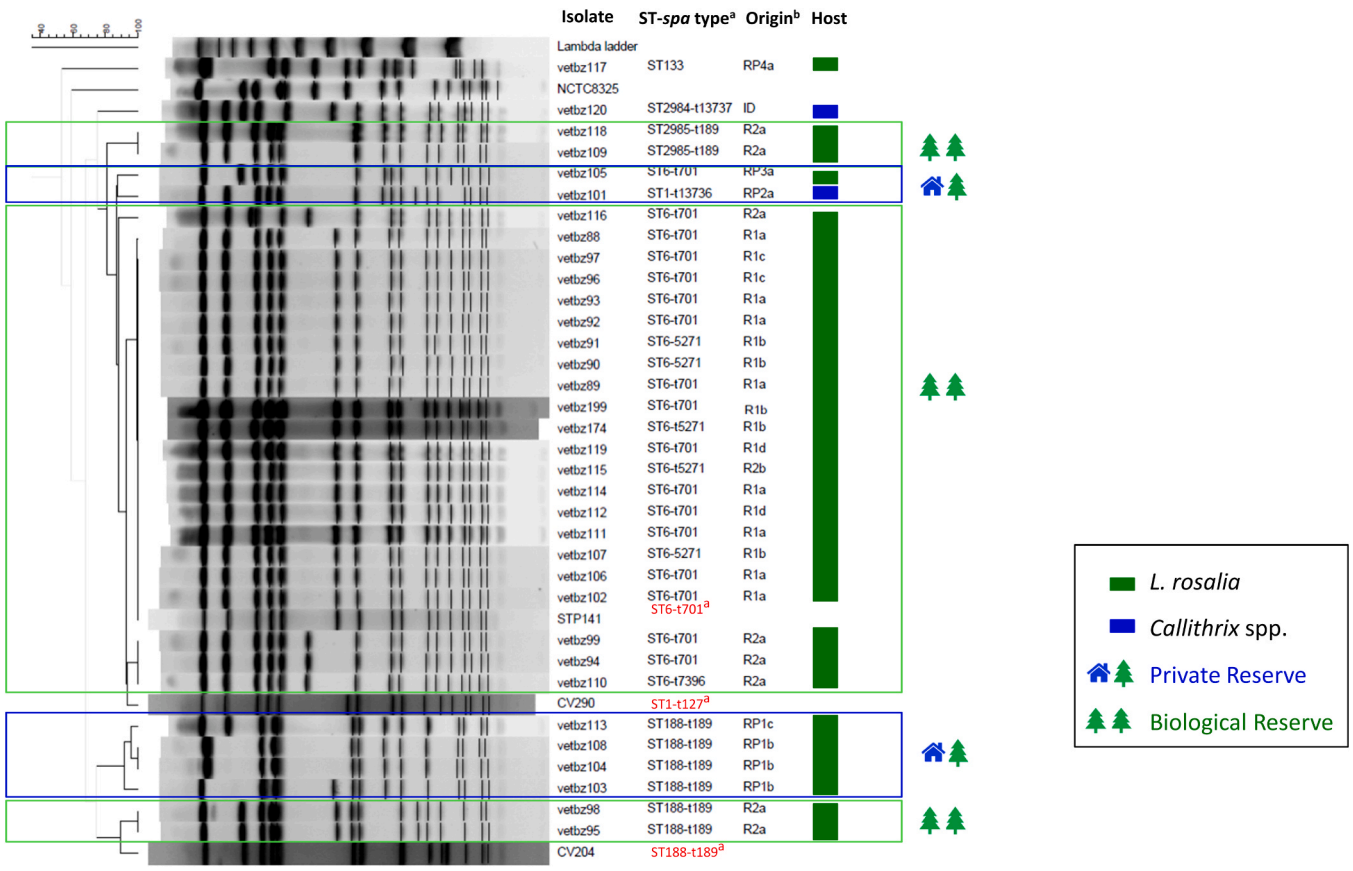

Fig. 4. *SmaI* PFGE profiles of *33 S. aureus* isolated from wild marmoset *(Callithrix* spp.) and golden lion tamarins (*L. rosalia*) in private and Biological Reserves in Brazil. A cut-off of 80 % similarity was used to define the PFGE types. Multilocus sequence type and *spa* type of each strain are indicated as well as the origin and host of the isolate. [a]Control strains; [b]Each distinct code corresponds to a different location in Biological (R) or private (RP) reserves.

Table 2
Genotypic and phenotypic characteristics of coagulase-negative staphylococci (CoNS) collected from non-human primates in environments with different levels of contact with humans.

Environment	Level of human contact	Host (no isolates)	Bacterial species (no isolates)	PFGE type (no isolates)	Antibiotic resistance (no isolates)
Private Reserve	Low	*L rosalia* (26)	*M. sciuri* (18)	X (2), C (1), E (1), H (1), I (3), J (5), L(1), M (1), Q (1)	Pen^R (3), Oxa^R (1), $Clin^R$ (4), Fus^R (18)
			S. simiae (7)	AA (3), DD (3), GG (1)	–
			S. saprophyticus (1)	b (1)	Fus^R
		Callithrix spp. (16)	*M. sciuri* (11)	B (2), U (1), E (2), G (2), F (1), A (2), V (1)	Pen^R (4), Fus^R (11)
			S. xylosus (4)	AAA (2), BBB (1), CCC (1)	Pen^R (2), Ery^R (3)
			S. saprophyticus (1)	a (1)	Fus^R (1)
Biological reserve	None	*L. rosalia* (10)	*S. simiae* (4)	FF (1), EE (1), BB (1), CC (1)	–
			M. sciuri (4)	P (1), ND (1), A (1), R (1)	Oxa^R (1), $Clin^R$ (3), Fus^R (4)
			S. xylosus (2)	DDD (1), EEE (1)	$Clin^R$ (1)

decreased susceptibility to clindamycin (1/6). Resistance to penicillin was observed in isolates collected from both *L. rosalia* and *Callithrix spp*; all found in private forest. Resistance to oxacillin was observed both in Private forests and Biological reserves in *L. rosalia*. In all CoNS strains presenting resistance to oxacillin and penicillin there was no detection of *mec*A/*mec*C and *bla*Z genes, respectively, either by PCR or Southern blotting. The great majority of the isolates showing phenotypic resistance to beta-lactams, but lacking *mecA*, belonged to the *M. sciuri* species (n = 8/10); the remaining isolates were *S. xylosus* (n = 2/10). Regarding the remaining staphylococci isolated, the only two *S. saprophyticus* isolates were resistant to fusidic acid and susceptible to the remaining antibiotics and *S. simiae* were susceptible to all antibiotics tested. Overall, the frequency of staphylococcal isolates showing resistance to at least one antibiotic was higher among *L. rosalia* from private forests (18/32, 56 %) than those from the biological reserves (5/37, 13 %) (p = 0.0003) (see Fig. 3B).

4. Discussion

We evaluated the colonization prevalence of virulent and antibiotic resistant staphylococci in an endangered protected NHP species – *L. rosalia* - and in introduced populations of hybrids of other NHPs - *Callithrix jacchus* and *penicillate* [20,49]. *L. rosalia* is a native species of the state of Rio de Janeiro, Brazil with populations occurring in government conservation units (biological reserves) and in neighboring forests in private lands, wherein the contact with humans, their animals or environments is higher. One of the results of human activities has been the introduction of *Callithrix* spp, into urban and industrial regions

(like Water Island) as well as into private forests, where they co-exist with *L. rosalia*.

Our results showed that the contact of *L. rosalia* and *Callithrix* spp. with human environments has led to changes in their microbiota and in their virulence genes content, and antibiotic susceptibility. *L. rosalia* from private forests were enriched in *M. sciuri* when compared to biological reserves and *Callithrix spp*. from Water Island were enriched in *S. aureus*, when compared to private forests. In particular, the frequency of *S. aureus* resistant to erythromycin and carrying PVL was higher in samples collected from *Callithrix* spp in the human environment, when compared to environments with less contact with humans. Furthermore, the frequency of resistance to at least one antibiotic (beta-lactams, macrolides and lincosamides) in the human-influenced environments (the island and the private forests) was higher for CoNS collected both from *L. rosalia* and *Callithrix spp.* The origin of such alterations is not clear but could have resulted from the contact of bacteria with environmental pressure associated to human activities, such as the contamination of water and/or soils with antibiotics, dissemination of antibiotic resistant bacteria from farms, the contact between the native and invader NHP species or the direct interaction of *Callithrix spp*. with humans in Water Island.

Penicillin, macrolides and lincosamides are extremely important antimicrobials for the treatment of infections in production animals (cattle and pigs) [50] and have been described to be important contaminants of farm waters and ground soil in farms [51]. Farming activities occur in the land surrounding the private forests in this region in Brazil and might be the source of antimicrobial resistant bacteria or of contamination of water and soil that could in its turn be the environmental pressure inducing the NHP microbiota change and the increased antimicrobial resistance observed.

Introduced *Callithrix spp* represent a risk for the transmission of potential pathogenic and antibiotic resistant *S. aureus to L. rosalia*, however our data did not support the existence of transmission of *S. aureus* between the two NHP species. On the other hand, the most important *S. aureus* clonal types found in NHP inhabiting the regions with the highest contact with humans, namely the private forests and Water island (ST398, ST188, ST133, ST1, ST6), were previously identified colonizing humans in Brazil [52]. It is possible that dissemination of *S. aureus* between humans and NHP, and not the dissemination between the two species of NHP, might have caused the observed increase in *S. aureus* in *Callithrix spp*. Other studies have documented the dissemination of antimicrobial resistant *S. aureus* between humans and NHP and PVL was previously found in *S. aureus* strains from NHP in captivity [15].

A somewhat different pattern was found for CoNS. The *M. sciuri* isolates collected from *Callithrix spp* and *L. rosalia* from the same geographic regions had closely related PFGE profiles. This suggests the dissemination of CoNS between the two primate hosts. Contrarily to what was observed for *S. aureus*, the results suggest that the contact between the native and invader NHP species could have caused the observed increase in *M. sciuri* prevalence and the occurrence of penicillin resistance among *L. rosalia* in private forests, where *Callithrix* also lives. A high resistance to penicillin was also previously found in staphylococci from vaginal mucosa of captive Azara's night monkey (*Aotus azarai infulatus*) and *L. rosalia* in Brazil [53,54].

In *Staphylococcus* the most widely spread mechanism of beta-lactams resistance is associated to *mecA* – the gene that encodes a penicillin binding protein with low affinity for beta-lactams (Pbp2A). In our study most of the *M. sciuri* isolates were resistant to beta-lactam antibiotics, but did not carry the *mecA* as detected by PCR and Southern blotting. This apparent discrepancy was previously described, and was proved to be due to the overexpression of *mecA1*, a *mecA* homolog, which is ubiquitous in *M. sciuri* and that is believed to be the *mecA* ancestral [26].

The extension of our findings is limited by the number of NHP that were sampled in the different types of reservations and the consequent low number of staphylococcal isolates of each species included in the study. The low number of NHP sampled could have impacted on the diversity of staphylococcal species and clonal types identified and on the number of epidemiological links found between staphylococci of the two species of primates. Sampling of wild animals is difficult and dependent on a number of uncontrollable factors, like the number of wild animals that occur in the specific sampling region in the specific sampling day. Even so, epidemiological links were established, and the conclusions taken from our study have statistical support. In this study transmission was assessed through PFGE, a high-resolution methodology used for many years as the state of the art method to assess bacterial transmission. Currently, with the advent of whole genome sequencing transmission events are usually detected by genomics approaches based on the analysis of single-nucleotide polymorphisms or core genome multilocus sequence typing that have higher resolution than PFGE. However, these are more appropriate to detect recent transmission events in closed environments like hospitals that presuppose a high similarity between strains, which is not the case of our study.

These results support the hypothesis that human activities and human contact influence the bacterial microbiota of wild and protected animals. We found evidence that *L. rosalia* in locations with increased human contact have a higher prevalence of colonization with antibiotic resistant and pathogenic bacteria than wild populations with reduced contact with humans.

Ethical statement

This study was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), certified by Authorization and Information Biodiversity System (SisBio) (protocol # 10596-2), and was approved by the Ethical Committee from Universidade Estadual do Norte Fluminense Darcy Ribeiro (protocol # 148). CEUA was approved in November, 2011, with four years validation.

Declaration of Competing Interest

No conflicts of interests to declare.

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