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MICROBIOLOGY

Isolation and antimicrobial resistance of coagulase-negative staphylococci recovered from healthy tortoises in Minas Gerais, Brazil

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ABSTRACT: *In the last few decades, there has been a global increase in the adoption of reptiles as companion animals, mainly turtles and tortoises. Considering the popularity of reptiles as pets in Brazil, and a notable lack of data about potentially pathogenic staphylococci in these animals, this study isolated and evaluate the antimicrobial susceptibility of staphylococcal species from healthy tortoises (Chelonoidis carbonaria) in Brazil. During a 12-month period (February 2019 to February 2020), cloacal swabs from 66 healthy tortoises were collected at the Wild Animals Screening Center in Belo Horizonte, Minas Gerais, Brazil. The swabs were plated onto mannitol salt agar for staphylococci isolation, and species identification was performed using MALDI-TOF MS. Antimicrobial susceptibility was investigated using the disk diffusion method, and the presence of the mecA gene was investigated by PCR to detect methicillin resistance. Of the tested animals, 72.7% were positive for staphylococcal isolation. All isolates were coagulase-negative staphylococci (CoNS), and Staphylococcus sciuri (81.3%), and S. xylosus (12.5%) were the most frequently isolated species. The majority of the isolates (56%) were resistant to at least one antimicrobial agent. A high frequency of resistance was observed for penicillin (35.5%) and tetracycline (29.1 %). All strains were susceptible to cefoxitin, chloramphenicol, ciprofloxacin, erythromycin, and gentamicin. All isolates were negative for the mecA gene. The present work suggests that healthy tortoises are mainly colonized by CoNS, especially S. sciuri. Half of the isolates were resistant to at least one antimicrobial, raising questions regarding the possible role of these animals as reservoirs of antimicrobial resistance genes.*
Key words: *reptiles, coagulase-negative staphylococci, S. sciuri, S. xylosus, Chelonoidis carbonaria.*

Isolamento e resistência aos antimicrobianos de estafilococos coagulase negativos recuperados de jabutis saúdáveis em Minas Gerais, Brasil

RESUMO: *Nas últimas décadas, houve um aumento global na adoção de répteis como animais de companhia, principalmente tartarugas e jabutis. Considerando a popularidade dos répteis como animais de estimação no Brasil e a notável falta de dados sobre estafilococos potencialmente patogênicos nesses animais, o objetivo deste estudo foi isolar e avaliar a susceptibilidade antimicrobiana de espécies estafilocócicas de jabutis (Chelonoidis carbonaria) saudáveis no Brasil. Durante um período de 12 meses (fevereiro de 2019 a fevereiro de 2020), suabes cloacais de 66 jabutis saudáveis foram coletados no Centro de Triagem de Animais Silvestres em Belo Horizonte, Minas Gerais, Brasil. Os suabes foram plaqueados em ágar manitol salgado para isolamento de estafilococos e a identificação das espécies foi realizada usando MALDI-TOF MS. A susceptibilidade aos antimicrobianos foi investigada pelo método de difusão em disco, e a presença do gene mecA foi investigada por PCR para detectar resistência à meticilina. Dos animais testados, 72,7% foram positivos para o isolamento estafilocócico. Todos os isolados eram estafilococos coagulase-negativos (CoNS), sendo Staphylococcus sciuri (81,3%) e S. xylosus (12,5%) as espécies mais frequentemente isoladas. A maioria dos isolados (56%) foi resistente a pelo menos um antimicrobiano. Alta frequência de resistência foi observada para penicilina (35,5%) e tetraciclina (29,1%). Todas as estirpes foram sensíveis à cefoxitina, cloranfenicol, ciprofloxacina, eritromicina e gentamicina. Todos os isolados foram negativos para o gene mecA. O presente trabalho sugere que jabutis saudáveis são colonizados principalmente por CoNS, especialmente S. sciuri. Metade dos isolados foram resistentes a pelo menos um antimicrobiano, levantando questões sobre o possível papel desses animais como reservatórios de genes de resistência aos antimicrobianos.*
Palavras-chave: *répteis, estafilococos coagulase-negativos, S. sciuri, S. xylosus, Chelonoidis carbonaria.*

INTRODUCTION

In the last few decades, there has been a global increase in the adoption of reptiles as companion animals. According to data issued by the latest census of companion animals, Brazil has the ninth largest population of reptiles in the world (IBGE, 2013). These exotic pets, especially turtles and tortoises, have gained popularity because of their appearance and complaisant nature (HOSSAIN et al., 2020).

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Despite the increasing popularity of reptiles as pets, studies have demonstrated that these animals represent a potential threat to public health (CDC, 2019; RAMOS et al., 2019). These animals can act as reservoirs and potential disseminators of pathogenic microorganisms, including *Salmonella* spp., pathogenic *Escherichia coli*, and *Leptospira* spp., and can also contribute to the spread of antimicrobial resistance genes (EBANI, 2017; RAMOS et al., 2019).

Staphylococcus spp. are important commensal bacteria, which can cause a wide variety of diseases in both humans and animals, including nosocomial infections (WIELER et al., 2011; WALTHER et al., 2017). The genus is divided into two groups according to production of the coagulase enzyme: coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS). CoPS are responsible for most infections, whereas CoNS represent most commensal staphylococci from different hosts and are involved in infections in a variety of species, including humans (DIMITRIOU et al., 2011; WALLER et al., 2011; BIEROWIEC et al., 2019). In addition to this pathogenic potential, CoNS have the ability to acquire, harbor, and transfer resistance genes to other staphylococci, and even to other bacterial species (OTTO, 2013; BECKER et al., 2014).

Over the years, CoPS and CoNS have been extensively investigated in humans and domestic animals but few studies have been performed in wildlife. There are reports of the colonization and antimicrobial resistance of staphylococci isolated from some wild mammals, birds, and even amphibians (SLAUGHTER et al., 2001; MAMA et al., 2019; RUIZ-RIPA et al., 2020). However, there are limited studies on staphylococci isolated from healthy reptiles, and most of these did not evaluate the antimicrobial resistance of the isolates (TADDEI et al., 2010; DI IANNI et al., 2015; ORIÁ et al., 2015; CARDOSO–BRITO et al., 2019).

Given the notable lack of data on the carriage and antimicrobial resistance profile of potentially pathogenic staphylococci in pet reptiles, this research evaluated the frequency, distribution, and antimicrobial-susceptibility patterns of staphylococcal species isolated from healthy tortoises in Brazil.

MATERIALS AND METHODS

Samples

A convenience sampling of 66 apparently healthy tortoises (*Chelonoidis carbonaria*) was conducted over a 12-month period (February 2019 to February 2020). Samples were collected by the veterinary staff of the Wild Animals Screening Center (CETAS) in Belo Horizonte city (Minas Gerais, Brazil), an agency responsible for receiving, rehabilitating, and reintroducing wild animals into their natural environment. The tortoises sampled in this research arrived at the center through voluntary delivery, rescue, or apprehension by surveillance agencies. For sampling procedures, a sterile swab (Cral; Cotia, SP, Brazil) was introduced 5–6 cm into the cloaca and rotated five times, as described by IVES et al. (2017). The swab was placed in a sterile microtube, stored in a transport box with ice packs, and transported to the Bacteriosis and Research Laboratory of the Veterinary School of the Federal University of Minas Gerais (UFMG) for immediate processing. The study was approved by the Ethical Committee on Animal Use (CEUA) of UFMG under protocol 238/2015 and by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) under protocol 49195-1.

Staphylococcus sp. isolation and identification

For *Staphylococcus* sp. isolation, swabs were plated onto Mannitol salt agar (Difco, USA) and incubated at 37 °C for 24 h. Morphologically suggestive colonies were isolated on Brain Heart Infusion agar (BHI) (Difco, USA) and identified by matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Bremen, Germany) using the standard extraction protocol recommended by Bruker (MATSUDA et al., 2012).

Antimicrobial susceptibility and mecA detection

Antimicrobial susceptibility tests were performed using disk diffusion in agar, according to the Clinical and Laboratory Standards Institute (CLSI) documents M100-S30 (CLSI, 2020) and VET08 (CLSI, 2018). The following antimicrobials were tested: cefoxitin (30 µg), penicillin (10 units), tetracycline (30 µg), trimethoprim/sulfamethoxazole (25 µg), chloramphenicol (30 µg), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10 µg), and ciprofloxacin (5 µg) (DME, BRA). *Staphylococcus aureus* ATCC 25923 was used as a control strain. Additionally, DNA extraction was performed, according to PITCHER, et al. (1989), and methicillin-resistant staphylococci (MRS) were investigated by detection of the *mecA* gene, as previously described by MURAKAMI et al. (1991).

Statistical analysis

The association between phenotypic resistance and staphylococcal species was evaluated

by chi-square or Fisher's exact tests, using GraphPad Prism v.8 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

RESULTS

In this study, 48 out of 66 (72.7%) tested tortoises were positive for staphylococcal species (Table 1). All isolates were CoNS and four different species were detected, with *S. sciuri* (81.3%) isolated significantly more frequently than the other species (Table 1) ($P < 0.001$). The frequency of *S. xylosus* was also significantly different from that of *S. kloosii* and *S. saprophyticus* ($P = 0.048$).

Twenty-seven (56.2%) isolates were resistant to at least one antimicrobial agent, while twenty-one (43.8%) were susceptible to all tested antimicrobials. There was a high frequency of resistance to penicillin G (35.5%), followed by tetracycline (29.1%), clindamycin (2%), and trimethoprim/sulfamethoxazole (2%) ($P < 0.05$). Resistance to penicillin G (CLI/SUT: $P < 0.001$; others: $P < 0.001$) and tetracycline (CLI/SUT: $P = 0.0004$; Others: $P < 0.001$) was significantly greater than that of the other tested antimicrobial agents. However, no significant differences were reported in resistance between penicillin G and tetracycline. All isolates were susceptible to cefoxitin, chloramphenicol, erythromycin, gentamicin, and ciprofloxacin.

Table 1 presents the antimicrobial resistance phenotypes of staphylococcal species recovered from healthy tortoises in Brazil. *Staphylococcus sciuri* presented resistance to penicillin G, tetracycline, and clindamycin. *S. xylosus* and *S. kloosii* presented resistance to penicillin G and tetracycline and *S. saprophyticus* presented resistance to tetracycline and trimethoprim-sulfamethoxazole. All four recovered staphylococcal species had isolates resistant to tetracycline, and all presented isolates with resistance to more than one antimicrobial agent (co-resistance). All isolates were negative for the *mecA* gene.

DISCUSSION

Despite the clear increase in the adoption of reptiles as pets, including tortoises, few studies have focused on the role of these species as a reservoir of bacterial pathogens. To the best of our knowledge, this is the first study to isolate staphylococci from healthy reptiles and investigate their antimicrobial susceptibility. Notably, the incidence of staphylococci in tortoises (72.7%) was similar to that in wild birds (60–75%) and wild mammals (37.7– 90.8%) (CHEN et al., 2016; SOUSA et al., 2016; MAMA et al., 2019; RUIZ-RIPA et al., 2020). These results suggested that staphylococci are commensals of the mucous membrane in tortoises, which is similar to previous observations in mammals and birds (NAGASE et al., 2002; SOUSA et al., 2016; RUIZ-RIPA et al., 2020).

All isolates in the present study were identified as CoNS (Table 1). There are limited data on the factors that influence colonization by CoNS. Recently, GARCÍA et al. (2020) suggested that CoPS colonization seems to be more frequent in carnivores, raising the hypothesis of an influence of diet on staphylococcal colonization in animals. Interestingly, tortoises are fed with non-meat-based diets, which could justify the presence of CoNS instead of CoPS isolates.

Staphylococcus sciuri was the predominant species recovered from the sample tortoises, accounting for more than 80% of the isolates (Table 1).

Table 1 - Frequency, distribution, and antimicrobial resistance phenotypes of staphylococcal species recovered from healthy tortoises (n = 66) in Minas Gerais, Brazil.

		----------Number of antimicrobial resistant isolates----------								
Species	n (%) *	PEN	TET	CLI	SXT	CEF	ERY	GEN	CIP	CLO
S. sciuri	39 (81.3) [a]	13	10	1	0	0	0	0	0	0
S. xylosus	6 (12.5) [b]	2	2	0	0	0	0	0	0	0
S. kloosii	2 (4.2) [c]	2	1	0	0	0	0	0	0	0
S. saprophyticus	1 (2) [c]	0	1	0	1	0	0	0	0	0
Total	48 (100%)	17 (35.5%)	14 (29.1%)	1 (2%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

*Multiple comparison: different letters indicate significant differences.
PEN, penicillin G; TET, tetracycline; CLI, clindamycin; SXT, trimethoprim-sulfamethoxazole; CEF, cefoxitin; ERY, erythromycin; GEN, gentamicin; CIP, ciprofloxacin; CLO, chloramphenicol.

This result was not surprising, as *S. sciuri* is the most common CoNS isolate in other healthy wild animals, including birds, boars, hedgehogs, red foxes, rabbits, badgers, and other mammals (SOUSA et al., 2016; MAMA et al., 2019; GARCÍA et al., 2020; RUIZ-RIPA et al., 2020). Furthermore, this bacterium is known to have a wide range of hosts, is adapted to different habitats (NEMEGHAIRE et al., 2014; GÓMEZ et al., 2017; SCHOENFELDER et al., 2017; RUIZ-RIPA et al., 2020), and has also been reported as the causative agent of occasional, but serious, infections in humans, including endocarditis, peritonitis, urinary tract infections, and septicemia (STEPANOVIĆ et al., 2002; STEPANOVIĆ et al., 2005; SEVERIN et al., 2010). In this context, it is important to remember that bilateral transmission of commensal staphylococci between pets and their owners has been widely reported (BAPTISTE et al., 2005; WEESE et al., 2006; HANSELMAN et al., 2009).

Staphylococcus xylosus, *S. kloosii*, and *S. saprophyticus* were also isolated in the present study; although, at a much lower frequency than *S. sciuri* (Table 1). These species are less pathogenic but opportunistic microorganisms, as are most CoNS (BECKER et al., 2014), and have been reported to colonize and cause infection in humans and some wild and domestic animals (SLAUGHTER et al., 2001; PEER et al., 2011; GIORDANO et al., 2016; SREDNIK et al., 2017). Specifically, *S. saprophyticus* is the second highest cause of urinary tract infections in women and is typically classified as a human colonizer (LATHAM et al., 1983; VON EIFF et al., 2002; RAZ et al., 2005; FARIÑA et al., 2013). Unfortunately, it is unclear if an anthropogenic activity and/or human contact, including manipulation of these animals, have had influenced the isolation rate of S. saprophyticus in the present study.

It is important to highlight that CoNS, including *S. sciuri*, are known for their capacity to carry and disseminate antimicrobial resistance determinants (SCHOENFELDER et al., 2017), which contribute to their pathogenic potential (STEPANOVIĆ et al., 2002; FREY et al., 2013; BEIMS et al., 2016). In the present study, more than half of the isolates were resistant to at least one tested antimicrobial agent, mostly penicillin G (35.5%) and tetracycline (29.1%) (Table 2), which are widely used in human and veterinary medicine. Unfortunately, the antimicrobial history of the sampled animals was unknown, but neither penicillin nor tetracycline are listed as common antimicrobials to treat bacterial infections in tortoises (MAUTINO & PAGE, 1993). In fact, the isolates were fully susceptible to most compounds recommended for these animals, including gentamicin, chloramphenicol, ciprofloxacin, and trimethoprim-sulfonamide.

Among the *S. sciuri* isolates, resistance to penicillin G and tetracycline (33.3% and 25.6%, respectively) was more common than resistance to the other antimicrobials tested. These rates were much lower than those reported by NEMEGHAIRE et al. (2014) (98.6% and 60%, respectively) and SCHOENFELDER et al. (2017) (74% and 87%, respectively) for *S. sciuri* isolates from farm animals. This result was expected since the selective pressure of antimicrobials is likely much lower in tortoises than that in livestock. The source of the resistance to penicillin and tetracycline in these animals is unknown, but both antimicrobials have moderate potential for bioaccumulation in plants (TASHO & CHO, 2016), which is the main food source for many animals, including captive and free-living tortoises.

Over the last decade, MRS have emerged, creating concern for human and veterinary health. Resistance to all beta-lactam antimicrobials as a consequence of the acquisition of the *mecA* gene excludes practically all of the first-choice treatment options for both animals and humans, drastically reducing therapeutic alternatives (MARIN, 2002). There are several reports on the colonization and infection of companion animals that transmit MRS

Table 2 - Antimicrobial resistance phenotypes of *Staphylococcus* species recovered from tortoises in Brazil.

Species	Total of isolates	Antimicrobial resistance phenotype[ab]	Co-resistance
S. sciuri	39	PEN[13], TET[10], CLI[1]	PEN-TET[2]; PEN-CLI[1]
S. xylosus	6	PEN[1]; TET[2]	PEN-TET[1]
S. kloosii	2	PEN[2]; TET[1]	PEN-TET[1]
S. saprophyticus	1	TET[1]; SXT[1]	TET-SXT[1]

[a]PEN, penicillin G; TET, tetracycline; CLI, clindamycin; SXT, trimethoprim-sulfamethoxazole.
[b]Superscript numbers indicate the number of isolates with the same characteristic.

strains (VAN DUIJKEREN et al., 2011; POMBA et al., 2017), showing the relevance of studies monitoring the occurrence of MRS in animals. Fortunately, there were no MRS isolates in the tortoises evaluated in this study. Although, previous studies have suggested that biofilms also play a role in the treatment resistance of staphylococci infection, information on biofilm production in CoNS isolated from wild animals is lacking (Lee et al., 2019). Thus, future studies should also evaluate the capacity of biofilm formation in these CoNS isolates from tortoises.

This study has some limitations. Due to the small number of sampled animals, further studies are needed to determine the prevalence of staphylococci and the most common species in tortoises. Additionally, other reptile species should be studied to better understand the prevalence of staphylococci in these animals, as well as to clarify their role as reservoirs of drug-resistant staphylococci.

CONCLUSION

In conclusion, the present research suggested that healthy tortoises are mainly colonized by CoNS, especially *S. sciuri*. Staphylococci from tortoises seem to be susceptible to the majority of antimicrobial classes tested, but more than half of the isolates were resistant to at least one antimicrobial, penicillin or tetracycline, both of which are widely used in veterinary medicine. This is the first study to evaluate the colonization and antimicrobial resistance profile of staphylococci isolated from the cloacae of healthy tortoises, as well as from healthy reptiles.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation and samples collection were performed by Nathalia Abreu Borges Trevizani and Angélica Maria Araújo Souza. Laboratory analysis were performed by Jordana Almeida Santana and Brendhal Almeida Silva. The first draft of the manuscript was written by Jordana Almeida Santana and Rodrigo Otávio Silveira Silva. All authors read and approved the final manuscript.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by the Ethical Committee on Animal Use (CEUA) of the Universidade Federal de Minas Gerais (UFMG) under protocol 238/2015 and by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) under protocol 49195-1.

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