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First report in South America of companion animal colonization by the USA1100 clone of community-acquired methicillin-resistant *Staphylococcus aureus* (ST30) and by the European clone of methicillin-resistant *Staphylococcus pseudintermedius* (ST71)

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Abstract

Background: Methicillin-resistant staphylococci can colonize and cause diseases in companion animals. Unfortunately, few molecular studies have been carried out in Brazil and other countries with the aim of characterizing these isolates. Consequently, little is known about the potential role of companion animals in transmitting these resistant bacteria to humans. In this work we searched for *mecA* gene among *Staphylococcus* isolates obtained from nasal microbiota of 130 healthy dogs and cats attended in a veterinary clinic located in the west region of Rio de Janeiro. The isolates recovered were identified to the species level and characterized using molecular tools.

Results: A community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolate related to USA1100 (Southwest Pacific clone) and susceptible to all non- β -lactams was detected in a cat (1.7%, 1/60). Another coagulase-positive isolate harboring *mecA* was recovered from a dog (1.4%, 1/70) and identified as *Staphylococcus pseudintermedius* (MRSP) related to the European clone (ST71). The two isolates of *Staphylococcus conhii* subsp. *urealyticus* (1.4%, 1/70 dogs and 1.7%, 1/60 cats), similarly to the MRSP isolate, also presented high-level multiresistance. The majority of the methicillin-resistant coagulase-negative staphylococci recovered were *Staphylococcus saprophyticus* (5.7%, 4/70 dogs and 6.7%, 4/60 cats) and all clustered into the same PFGE type. (Continued on next page)

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Conclusions: This work demonstrates that *mecA*-harboring *Staphylococcus* isolates are common members of the nasal microbiota of the healthy companion animals studied (9.2%, 12/130 animals), including some high-level multiresistant isolates of *S. pseudintermedius* and *S. conhii* subsp. *urealyticus*. The detection, for the first time in South America, of USA1100-related CA-MRSA and of ST71 MRSP (European clone), colonizing companion animals, is of concern. Both *S. pseudintermedius* and *S. aureus* are important agents of infections for animals. The USA1100 CA-MRSA is a causative of severe and disseminated diseases in healthy children and adults. Additionally, MRSP is a nosocomial pathogen in veterinarian settings. It had already been demonstrated that the virulent ST71 MRSP is geographically spread over Europe and USA, with potential for zoonotic infections.

Keywords: CA-MRSA, Companion animals, *S. aureus*, Clonality, MRCoNS, *S. pseudintermedius*, *S. saprophyticus*

Background

The end of the 1980 decade was marked by the emergency of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) among human populations from a remote area in Australia [1]. The first strain reported was named Western-Australia 1 (WA-1) and later genotyped as ST1-SCC*mec* IV. WA-1 isolates did not frequently carry the genes *lukSF*, encoding for the two subunits (S and F) of the Pantone-Valentine leukocidin (PVL) [1,2]. Soon after, CA-MRSA clones spread over different countries [3]. In the USA, three main PVL-positive CA-MRSA clones have been prevailing: USA 300 (ST8-SCC*mec* IV), USA 400 (ST1-SCC*mec* IV) and USA1100 (ST30-SCC*mec* IV), with USA 300 isolates (ST8-SCC*mec* IV, PVL⁺) ranking first among the causatives of skin/soft skin infections in healthy individuals from the community [4-6]. In Brazil, there are few reports on CA-MRSA infections and most of the isolates are related to USA 1100, which have been detected causing infections ranging from simple and localized to severe and invasive diseases, in previously healthy children and adults [7]. More recently, MRSA isolates have been found colonizing and infecting both companion and livestock animals with clear economical and public health implications [8-10]. The detection of MRSA in animals has raised concerns among scientists over their role as potential reservoirs or vectors for CA-MRSA infections in humans. Besides MRSA, other methicillin-resistant staphylococci (MRS) have recently been detected among companion animals such as methicillin-resistant *Staphylococcus pseudintermedius* (MRSP). *S. pseudintermedius* is a major cause of purulent and opportunistic infections in dogs, such as dermatitis, otitis and wound infections. Indeed, MRSP are frequently multidrug resistant pathogens resembling typical hospital isolates [11,12].

Methicillin-resistant coagulase negative staphylococci (MRCoNS) have also been isolated in low frequencies from small companion animals and horses [13,14]. *Staphylococcus saprophyticus*, for example, is a common agent of uncomplicated urinary tract infection (UTI) in young women [15]. Although the report of *mecA*-harboring *S. saprophyticus* is very rare, these isolates have

recently been detected from human patients presented with UTI [16,17]. Besides, MRCoNS are recognized as opportunistic pathogens for immunocompromized patients, including high-risk human neonates [18]. Of concern is the fact that MRCoNS are considered a potential reservoir of resistance determinants, including staphylococcal cassette *mec* [17]. In the study presented here we searched for *mecA*-harboring *Staphylococcus* isolates in the nasal microbiota of 130 companion animals attended in a veterinary clinic in Rio de Janeiro. The isolates recovered were identified at subspecies level, and the antimicrobial susceptibility and genotypic profiles determined.

Results and discussion

The *mecA* gene was confirmed in the 12 staphylococci that grew in the enrichment broth (9.2%, 12/130 animals). From these methicillin-resistant streptococci (MRS) isolates 10 were MRCoNS (7.7%, 10/130) and 2 coagulase-positive MRS (1.5%, 2/130). The majority of MRCoNS were identified as *Staphylococcus saprophyticus* (80%, 8/10) and the remaining as *Staphylococcus conhii* ssp. *urealyticus* (20%, 2/10). One coagulase-positive isolate (BMBSA87), collected from a cat, harbored *mecA* and was identified as MRSA (8.3%, 1/12) and the other isolate (BMBSP02), obtained from a dog, was initially identified as *Staphylococcus intermedius* by the automated method (8.3%, 1/12). Because all dog isolates identified thus far by routine phenotypic tests as *S. intermedius* clustered into the newly identified species *S. pseudintermedius* [19], additional PCR identification was carried out yielding an expected single band of 926 bp, confirming the classification of this isolate as *S. pseudintermedius*.

The MRSA isolate was susceptible to all drugs tested except β -lactams. All isolates identified as *S. saprophyticus* (except one that was also resistant to rifampicin; 7/8; 87.5%) displayed antimicrobial resistance only to erythromycin and clindamycin, in addition to β -lactams. Despite that, the MRSP isolate was resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin and trimethoprim sulphamethoxazole, in addition to β -

lactams. Multiresistance was also detected in 2 isolates of *S. conhii* ssp. *urealyticus* (Table 1).

The prevalence of MRS among dogs was as follows: 1.4% (1/70) carried MRSP, 1.4% (1/70) methicillin-resistant *S. conhii*-urea (MRSC) and 5.7% (4/70) methicillin-resistant *S. saprophyticus* (MRSS), and among cats was: 1.7% (1/60) carried MRSA, 1.7% (1/60) MRSC and 6.7% (4/60) MRSS. The MRSA isolate BMBSA87 was typed as SCCmec IV and displayed a PFGE pattern indistinguishable to that of CA-MRSA isolate WB49, a representative of the USA1100 clone (ST30-SCCmec IV; Figure 1A, Lanes 5–6), previously detected in Porto Alegre, RS, Brazil, from human skin/soft skin infection [20]. In addition, isolate BMBSA87 displayed the multilocus sequencing type (MLST) allelic profile: arcc = 2, aroe = 2, glpf = 2, gmkn_ = 2, pta_ = 6, tpi_ = 3, yqil = 2, corresponding to ST30. However, amplification for the genes *lukSF* (encoding for PVL) could not be detected when total DNA of this isolate was tested with two different, specific, set of primers. PVL is commonly produced by ST30-SCCmec IV isolates [21,22]. Despite that, cases of skin/soft tissue infections (SSTI) associated with PVL-negative CA-MRSA isolates have been reported [22,23]. In addition, the MRSA isolate detected in the present study harbored *pmsa3* encoding for the phenol soluble moduline α 3, which has been associated with the pathogenesis of SSTI [24]. Our data showed that this MRSA isolate also carried the enterotoxin genes *seg*, *sei*, *seo* of the *egc* cluster, a highly prevalent operon of enterotoxin genes. It was suggested that the apparent redundancy of these superantigens provides

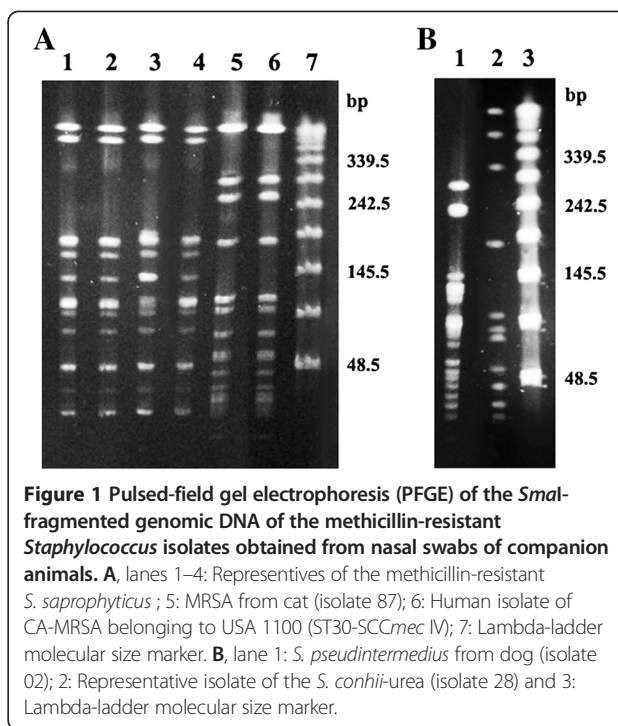


Figure 1 Pulsed-field gel electrophoresis (PFGE) of the *SmaI*-fragmented genomic DNA of the methicillin-resistant *Staphylococcus* isolates obtained from nasal swabs of companion animals. **A**, lanes 1–4: Representatives of the methicillin-resistant *S. saprophyticus*; 5: MRSA from cat (isolate 87); 6: Human isolate of CA-MRSA belonging to USA 1100 (ST30-SCCmec IV); 7: Lambda-ladder molecular size marker. **B**, lane 1: *S. pseudintermedius* from dog (isolate 02); 2: Representative isolate of the *S. conhii*-urea (isolate 28) and 3: Lambda-ladder molecular size marker.

selective advantage towards bacterial colonization and/or spread of the host and not only for toxemia [25]. The rate of MRSA colonization in dogs (1.4%, 1/70) was similar to those obtained in other studies (about 1- 2%) carried out in Hong Kong (2.1%, 15/704) and UK (0.7%, 6/850) [26,27]. Indeed, small number of MRSA was

Table 1 Species identification and antimicrobial susceptibility profiles of methicillin-resistant *Staphylococcus* isolated from healthy companion animals

Isolate	Species	Animal	Antimicrobial susceptibility ^a												
			CEF	CIP	CLI	CLH	ERY	GEN	OXA	PEN	RIF	TEI/ VAN	TET	TMP	
BMBS02	<i>S. pseudintermedius</i>	Dog	R ^b	R	R	R	R	R	R	R	R	S	S	S	R
BMBSA87	<i>S. aureus</i> ^c	Cat	R	S	S	S	S	S	R	S	S	S	S	S	S
BMBS028	<i>S. conhii</i> -urea ^d	Cat	R	S	R	S	R	R	R	R	R	S	S	S	R
BMBS030	<i>S. conhii</i> -urea	Dog	R	R	R	R	R	R	R	R	R	S	S	S	S
BMBS005	<i>S. saprophyticus</i>	Dog	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS018	<i>S. saprophyticus</i>	Cat	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS021	<i>S. saprophyticus</i>	Dog	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS035	<i>S. saprophyticus</i>	Cat	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS106	<i>S. saprophyticus</i>	Cat	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS110	<i>S. saprophyticus</i>	Cat	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS116	<i>S. saprophyticus</i>	Dog	R	S	R	S	R	S	R	R	R	S	S	S	S
BMBS130	<i>S. saprophyticus</i>	Dog	R	S	R	S	R	S	R	R	R	S	S	S	S

^aAntibiograms were carried out as described by the CLSI for the following antimicrobial drugs: cefoxitin (CEF), ciprofloxacin (CIP), clindamycin (CLI), chloramphenicol (CHL), erythromycin (ERY), gentamicin (GEN), penicillin G (PEN), rifampicin (RIF), sulfamethoxazole-trimethoprim (TMP), teicoplanin (TEC) and tetracycline (TET). All isolates were susceptible to vancomycin (VAN) when MIC was determined.

^bR: resistant and S: susceptible.

^c*S. aureus*: *Staphylococcus aureus* ssp. *aureus*.

^d*S. conhii*-urea: *S. conhii* ssp. *urealyticus*.

detected as cause of urinary tract infections in dogs in Brazil (0.9%, 3/348) [13]. This incidence is in accordance with previous data reporting low percentage (1.2%) of CA-MRSA carriers among human infant in Brazil [28].

S. pseudintermedius is an important opportunistic pathogen of companion animals. It is the most frequent *Staphylococcus* isolates collected from infected dogs. Since 2006 there has been a significant emergence of MRSP [12]. Many of MRSP isolates from Europe and USA (about 54%) were classified as ST71-SCC*mec*II, III or NT (NT: nontypable) [11,12]. The MRSP recovered here from dog (isolate BMBSP02) was typed as SCC*mec* NT. Additionally, the lambda-ladder molecular size marker was used as reference to calculate the sizes of the PFGE bands (Figure 1B) of the isolate BMBSP02. These values were compared with those obtained from PFGE images of the MRSP isolates published by Ruscher et al. [12] and Soedarmanto et al. [29], and it was found that the banding patterns were quite similar. Thus, MLST analysis was then carried out and confirmed the presence of this international virulent MRSP clone (European clone) in our country, since the BMBSP02 clustered into ST71, corresponding to the MLST allelic profile *ack* = 3, *cpn60* = 9, *fdh* = 1, *pta* = 2, *sar* = 2, *tuf* = 1. In Brazil, oxacillin resistance had already been described among *S. pseudintermedius* isolates causing urinary tract infections (8.6%, 6/70 dogs) and external otitis (14.3%, 13/91 dogs), on the basis of oxacillin 1 µg disk. However, these isolates were not fully molecular characterized [13,30]. In a large study, carried out in two distinct localities in South China, with 785 infected or colonized companion animals (612 dogs and 173 cats), it was found a total of 8.8% (69/785) of MRSP, which represented 47.9% (69/144) of the total *S. pseudintermedius* isolates [31]. It is impressive the high level of multiresistance showed by MRSP isolates. In fact, increased antibiotic resistance has recently been a tendency among MRSP isolates of the international clonal complex [12] and cross-transmission dog-owner of a multiresistant MRSP has already been reported [29].

All eight MRSS isolates, from both dogs and cats, clustered into the same PFGE clone type (Figure 1A; lanes 1–4) and generated amplification products for both DCS and *mecI* primers described in 2007 by Milheiro et al. [32] (Figure 2). These data indicated that the genes around *mec* complex were similar to those of SCC*mec* II and III (type II-III). Interestingly, eight MRSS isolates detected in Japan, which were nontypeable by the current classification for MRSA or by SCC*mec* sequencing, had the novel *mec* type II-III [16]. Despite the fact that MRSS isolates were the most frequent MRS colonizing both cats and dogs in our study, *S. saprophyticus* have been isolated in low frequency from infected cats and dog [33]. It is well known that in young women, *S. saprophyticus* is a common agent of urinary tract infections [15], but still its relative importance is low compared with *E. coli* [34]. In a study

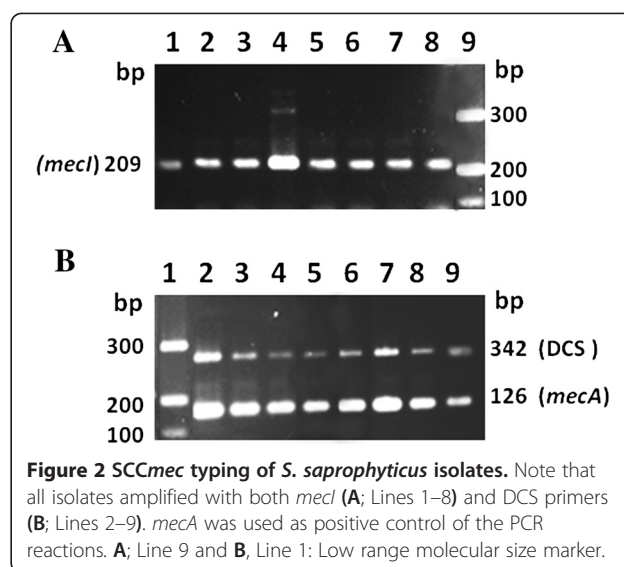


Figure 2 SCC*mec* typing of *S. saprophyticus* isolates. Note that all isolates amplified with both *mecI* (A; Lines 1–8) and DCS primers (B; Lines 2–9). *mecA* was used as positive control of the PCR reactions. A; Line 9 and B, Line 1: Low range molecular size marker.

conducted during one year in Sweden, with women and men of all ages, growth of *S. saprophyticus* was a quite uncommon finding [34]. The majority of individuals with *S. saprophyticus* were women between 15–29 years old (63.8%). However it is important to mention that, in this group, *S. saprophyticus* constituted 12.5% of all urinary tract pathogens [34]. It is also of significance that although still rare, isolates of MRSS have already been reported infecting humans in few countries as Canada [35], USA [36], Japan [16], Sweden [17], and also Brazil [37].

The PFGE type of a representative of *S. conhii* ssp. *urealyticus* (*S. conhii*-urea) is presented in Figure 1B (lane 2). Similarly to the MRSP isolate, *S. conhii*-urea isolates could not be typed for SCC*mec* with the type system used. Although very rare and generally considered a commensal bacteria, the presence of *mecA* has already been described in *S. conhii* either from human or animal origins [38].

Conclusions

This work demonstrates that *mecA*-harboring *Staphylococcus* isolates are common members of the nasal microbiota of the companion animals examined. Some bacterial species recovered are involved not only in animals but also in human infections. Moreover, the presence of USA1100-related MRSA colonizing animals is of concern. ST30 isolates are important CA-MRSA pathogens worldwide spread that has been associated to serious invasive diseases in adult and children in this country [39]. *S. pseudintermedius* is the most prevalent pathogenic streptococci in companion animals. To the best of our knowledge, that is the first report recognizing the prevalence of the European clone of MRSP (ST71) and of the USA1100 clone of MRSA (ST30) colonizing animals in Brazil. Both CA-MRSA and MRSP have already been incriminated in potential zoonotic

diseases, reflecting the recent emergency of these important multidrug-resistant pathogens in animals.

The fact that most of *mecA*-positive isolates were *S. saprophyticus* also poses an important question concerning potential transfer of MRSS between animals and humans. Finally, our data indicate that continued surveillance studies involving fully identification and genotyping of *Staphylococcus* isolates should be carried out in this and other countries for monitoring the spread of MRS among companion animals.

Methods

Sample collection

Hundred and thirty nasal swabs were obtained from 70 dogs and 60 cats which were visiting a veterinarian clinic, located in Rio de Janeiro city, RJ, for pet grooming, during the year of 2010. The animals included in this study were healthy and free of hospitalization, antimicrobial therapy and invasive device in the six-month before sample collections. The swabs were collected from the anterior nares of the animals, using a transport system (Culture Swab Transport System; Copan Italia SpA.; Brescia Italy), and stored at 4°C for no more than 24 h. Written informed consent for participation was obtained from the owners of all animals included in the study. The study was approved (# IMPPG030) by the Animal Ethics Committee from Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro.

Bacterial identification

In the laboratory, the swab was introduced in an enrichment broth constituted of tryptic soy broth (TSB, BD; Becton, Dickinson and Company, BD; Becton Drive-Franklin Lakes, NJ, USA) supplemented with 10 µg/mL methicillin and 7.5% of NaCl [40]. After incubation at 37°C for 18 h-48 h, aliquots of the turbid cultures were streaked on mannitol salt agar (BD; Becton, Dickinson and Company, BD; Becton Drive Franklin Lakes, NJ, USA) and incubated at 37°C for 18 h-24 h. Tube coagulase test was carried out for Gram-positive and catalase-positive colonies. Both coagulase-positive and -negative staphylococci were identified using Microscan WalkAway 96 SI automated system (Siemens Healthcare Diagnostic, West Sacramento, CA, USA) using Pos Combo Panel Type 33 (PC33) for bacterial identification and susceptibility tests. PCR amplification using the set of primers pse-F2 TRGG CAGTAGGATTCGTAA and pse-R5 CTTTTGTGCTY CMTTTTGG was carried out for molecular identification of *S. pseudintermedius*, as previously described [41].

Antimicrobial susceptibility testing

Antibiograms were also carried out following the Clinical Laboratory Standard Institute (CLSI) [42] recommendations for the following antimicrobial disks: cefoxitin (30 µg;

CEF) ciprofloxacin (5 µg; CIP), clindamycin (2 µg; CLI), chloramphenicol (30 µg; CHL), erythromycin (15 µg; ERY), gentamicin (10 µg; GEN), penicillin G (10 UI; PEN), rifampicin (5 µg; RIF), sulfamethoxazole-trimethoprim (25 µg; TMP), teicoplanin (30 µg; TEC) and tetracycline (30 µg; TET). The minimum inhibitory concentration (MIC) was determined for vancomycin (Sigma-Aldrich, St. Louis, MO, USA) according to CLSI guidelines [42]. The *mecA* was tested by polymerase chain reaction (PCR)-amplification of an internal fragment of the gene [43]. *Staphylococcus aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used for quality control purposes.

Bacterial genotyping

SCC*mec* typing was carried out for all isolates using different typing systems [32,43-46]. Pulsed-field gel electrophoresis (PFGE) of the *Sma*I-fragmented DNA was carried out for all isolates recovered, according to Teixeira et al. [47]. Multilocus sequence typing (MLST) was carried out as recommended for MRSA [48] and *S. pseudintermedius* [49] isolates. To assign the sequence types the allele sequences were correctly trimmed and submitted to the *S. aureus* MLST database (<http://www.mlst.net>), for the CA-MRSA BMBSA87, or to the MLST website (<http://pubmlst.org/spseudintermedius/>) sited at the University of Oxford for *S. pseudintermedius* [50].

Detection of virulence genes

The gene *lukF-PV* and those encoding for enterotoxins G, I, N and O of the *egc* cluster (*seg*, *sei*, *sen* and *seo*) were assessed by PCR, as described previously [20]. Additionally, another set of primers was used to test the genes *lukSF-PV* [21]. The *pmsα3* was tested with the primers described by Li et al. [25].

Abbreviations

CA-MRSA: Community-acquired methicillin-resistant *Staphylococcus aureus*; CEF: Cefoxitin; CIP: Ciprofloxacin; CHL: Chloramphenicol; CLI: Clindamycin; CLSI: Clinical Laboratory Standard Institute; CoNS: Coagulase-negative staphylococci; *egc*: Enterotoxin gene cluster; ERY: Erythromycin; GEN: Gentamicin; MLST: Multilocus locus sequence typing; MRS: Methicillin-resistant staphylococci (MRS); MRSA: Methicillin-resistant *Staphylococcus aureus*; MRCoNS: Methicillin-resistant coagulase negative staphylococci; MRSC: Methicillin-resistant *Staphylococcus conorii* ssp. *urealyticus*; MRSP: Methicillin-resistant *Staphylococcus pseudintermedius*; MRSS: Methicillin-resistant *Staphylococcus saprophyticus*; PCR: Polymerase chain reaction; PEN: Penicillin G; PFGE: Pulsed-field gel electrophoresis; PVL: Panton-Valentine leukocidin; RIF: Rifampicin; SCC*mec*: Staphylococcal cassette chromosome *mec*; ST: Sequence Type; SSTI: Skin/soft tissue infections; TEC: Teicoplanin; TET: Tetracycline 30 µg; TMP: Sulfamethoxazole-trimethoprim; TSB: tryptic soy broth; UTI: Urinary tract infections; WA-1: Western-Australia 1.

Competing interests

All authors declare that they have no competing interest.

Authors' contributions

IMZQ, MSR, MCSC and LCR carried out the bacterial isolation, identification and participate in the majority of the experiments of this paper. COB and RRS carried out PFGE analysis. TFO has been involved in the study design and in the draft of the manuscript. RO was responsible for examining and

selecting healthy animals, for collecting bacterial specimens and also for revising critically the manuscript with important intellectual contributions. PDP carried out experiments for identification and speciation of the coagulase-negative isolates. AMSF has been involved in the study design and in the revision of the manuscript critically for final approval of this version. All authors read and approved the final manuscript.

Acknowledgments

This work was supported in part by grants of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and European Commission's Seventh Framework Programme (FP7), through the Marie Curie International Research Staff Exchange Scheme NANO_GUARD (PIRSES-GA-2010-269138).

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Received: 28 March 2013 Accepted: 19 August 2013

Published: 27 August 2013

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doi:10.1186/1756-0500-6-336

Cite this article as: Quitoco et al.: First report in South America of companion animal colonization by the USA1100 clone of community-acquired methicillin-resistant *Staphylococcus aureus* (ST30) and by the European clone of methicillin-resistant *Staphylococcus pseudintermedius* (ST71). *BMC Research Notes* 2013 **6**:336.

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