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ORIGINAL ARTICLE

Methicillin-resistant *Staphylococcus aureus* of lineage ST398 as cause of mastitis in cows

N.C.C. Silva[1,2], F.F. Guimarães[3], M.P. Manzi[3], A. Fernandes Júnior[1], E. Gómez-Sanz[4,5], P. Gómez[4], H. Langoni[3], V.L.M. Rall[1] and C. Torres[4]

1 Department of Microbiology and Immunology, UNESP, Botucatu, SP, Brazil
2 Department of Agri-food Industry, Food and Nutrition - LAN, USP, Piracicaba, SP, Brazil
3 Department of Hygiene Veterinary and Public Health, UNESP, Botucatu, SP, Brazil
4 Biochemistry and Molecular Biology Area, University of La Rioja, Logroño, Spain
5 Environmental Genomics and Systems Biology Research Group, Institute of Natural Resource Sciences, Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland

Significance and Impact of the Study: Few studies on the epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) from bovine isolates have been performed in Brazil. MRSA of lineage ST398 is worldwide spread and associated with farm animals. Multidrug-resistant MRSA-ST398 isolates were recovered in 11% of mastitic cows from a single farm, with one isolate carrying the unusual *lsa(*E), *spw* and *aad*E genes. To our knowledge, this is the first detection of MRSA-ST398 isolates in milk samples of cows with mastitis in Brazil.

Keywords
bovine, *mec*A, methicillin-resistant *Staphylococcus aureus*, *Staph. aureus*, ST398, t011.

Correspondence
Carmen Torres, Universidad de La Rioja, Área de Bioquímica y Biología Molecular, Madre de Dios 51, 26006 Logroño, Spain.
E-mail: carmen.torres@unirioja.es

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Abstract

The objective of this study was to analyse the prevalence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) in milk of cows with mastitis. The California mastitis test (CMT) was used to detect the presence of mastitis in all 100 cows of a farm in Brazil. The CMT was positive in milk of 115 mammary quarters from 36 cows (36%). MRSA isolates were recovered from 4 of these 36 cows with mastitis (11%), and they were further characterized (one MRSA/sample). The four MRSA isolates were typed as t011-ST398-*agr*1-*SCCmec*V and presented two different pulsed-field-gel-electrophoresis-ApaI patterns. These four MRSA isolates showed resistance to tetracycline, streptomycin and ciprofloxacin, carried the *mec*A, *bla*Z, *tet*(K), and *tet*(M) resistance genes, and presented the S84L and S80F amino acid substitutions in GyrA and GrlA proteins, respectively. Two ST398 isolates exhibited resistance to gentamicin and tobramycin [with *aac*(6)-*aph*(2") and *ant(4)-Ia* genes] and one isolate resistance to clindamycin [with *lnu*(B) and *lsa* (E) genes]; this latter isolate also carried the spectinomycin/streptomycin resistance genes *spw* and *aad*E. MRSA of lineage ST398 is worldwide spread, normally multidrug resistant and may be responsible for bovine mastitis. To our knowledge, this is the first detection of MRSA-ST398 in Brazil.

Introduction

Bovine mastitis sums a significant economic impact on the dairy industry. *Staphylococcus aureus* is considered one of the most important pathogens in bovine clinical and subclinical mastitis (Kumar *et al.* 2011). The expression of the *mec*A gene in *Staph. aureus* confers resistance to most of β-lactams, including methicillin resistance (MRSA), agents frequently used for treatment of mastitis (Sawant *et al.* 2005). MRSA is an important human and animal pathogen that can be implicated in a wide diversity of infections, including bovine mastitis (Stefani *et al.* 2012).

MRSA of the sequence type ST398 is considered an important livestock-associated lineage, mainly related to

pig farming (Fluit 2012). This lineage has also been detected as colonizers or as causative agent of infection in other animal species, as bovine, equine, poultry and dogs in different countries (Nemati *et al.* 2008; Van den Eede *et al.* 2009; Floras *et al.* 2010; Feßler *et al.* 2012; Gómez-Sanz *et al.* 2013), as well as in humans (Lozano *et al.* 2011b). However, MRSA ST398 has never been detected, to our knowledge, in Brazil. The objective of this study was to analyse the proportion of MRSA in milk of cows with mastitis in a Brazilian farm and to perform the molecular typing and the genetic characterization of the MRSA isolates obtained.

Results and discussion

The California mastitis test (CMT) was used to detect the presence of clinical or subclinical mastitis in all 100 cows of a farm in Brazil and was applied to 400 samples of milk of these animals (one milk sample per mammary gland). The CMT was positive in 115 mammary quarters of 36 cows (36% of cows). Milk bacterial culture from these 115 mammary quarters yielded 32 staphylococcal intramammary infections (IMI) coming from 18 cows. *Staph. aureus* was recovered from 15 mammary quarters from 8 cows. Among the *Staph. aureus* recovered, MRSA was detected in 4 mammary quarters from 4 cows, representing 4% of cows and 11·1% of cows with mastitis, as defined by CMT. The four MRSA isolates (one per positive animal) were further characterized (Table 1).

The 4 MRSA isolates were typed as *spa*-type t011, which is associated to lineage ST398, of clonal complex CC398, presented the *agr* type I and the SCC*mec* type V. The PFGE profile of the strains exhibited two different patterns (C6129 and C6130 pattern A; C5960 and C6128 pattern B) (Table 1). All isolates were negative for the toxin genes *lukF/lukS*, *tst*, *eta* and *etb* as well as for the genes of the immune evasion cluster (IEC). All isolates exhibited a multidrug-resistant phenotype (including at least three classes of antimicrobial agents). In addition to β-lactams, isolates showed resistance to tetracycline, streptomycin and ciprofloxacin (100%), gentamicin and tobramycin (50%), and clindamycin (25%). The resistance genes detected in these isolates are shown in Table 1. The genetic determinant for streptomycin resistance (*aadE*) was observed in a single strain (C6129) (Table 1). In addition, this isolate harboured the clindamycin resistance gene *lnu*(B) and the recently described *lsa*(E). These genes (*lsa*(E), *aadE* and *spw*) were enclosed within the same antimicrobial gene cluster, which shared structural homology to the one recently described by other authors (Lozano *et al.* 2012a; Wendlandt *et al.* 2013a,b). Amino acid changes in GyrA (S84L) and in GrlA (S80F) were identified in the 4 isolates (Table 1).

Few studies have detected MRSA ST398 in milk from mastitic cows (Feßler *et al.* 2010; Vanderhaeghen *et al.* 2010). MRSA ST398 has gained special attention as colonizers and causative agents of infections in pigs (Gómez-Sanz *et al.*2010; Fluit 2012). Further reports have shown that ST398 isolates are not restricted to these animals, but can be also isolated from humans, bovines, poultry, horses and dogs (Nemati *et al.* 2008; Van den Eede *et al.* 2009; Feßler *et al.* 2010; Floras *et al.* 2010; Lozano *et al.* 2011b; Fluit 2012). MRSA of this lineage is considered an important zoonotic clone, given that transmission between different animal species and humans has been suggested in numerous occasions (Lozano *et al.* 2011a; Feßler *et al.* 2012; Fluit 2012). MRSA ST398 seems to be an important cause of mastitis in cows of the studied farm, as 11·1% of tested animals were diagnosed with mastitis. All our MRSA ST398 isolates were ascribed to

Table 1 Characterization of the MRSA isolates recovered from mastitic cows in this study

Isolate number	*spa*-MLST	PFGE profile	*agr* type	SCC*mec* type	Resistance phenotype	Resistance genes detected	Amino acid substitutions within the QRDR* of	
							GyrA	GrlA
C5960	t011-ST398	A	I	V	PEN, OXA, FOX, TET, STR, CIP	*bla*Z, *mec*A, *tet*(K), *tet*(M)	S84L	S80F
C6128	t011-ST398	A	I	V	PEN, OXA, FOX, TET, STR, CIP	*bla*Z, *mec*A, *tet*(K), *tet*(M)	S84L	S80F
C6130	t011-ST398	B	I	V	PEN, OXA, FOX, TET, TOB, GEN, STR, CIP	*bla*Z, *mec*A, *tet*(K), *tet*(M), *ant*4, *aac(6′)-aph(2″)*	S84L	S80F
C6129	t011-ST398	B	I	V	PEN, OXA, FOX, TET, TOB, GEN, STR, CIP, CLI	*bla*Z, *mec*A, *tet*(K), *tet*(M), *ant*4, *aac(6′)-aph(2″)*, [*lnu*(B), *lsa*(E), *spw, aadE*]†	S84L	S80F+R44H

PEN, penicillin, OXA, oxacillin, FOX, cefoxitin, TET, tetracycline, CLI, clindamycin, TOB, tobramycin, GEN, gentamicin, STR, streptomycin, CIP, ciprofloxacin.

*Quinolone resistance determining region.

†Genes expected to be physically linked based on PCR mapping and bibliography.

the *spa*-type t011, widely distributed among MRSA ST398 from pigs (Gómez-Sanz *et al.* 2010). In our study, CMT was used as predictor test of clinical and subclinical mastitis and only those CMT-positive milk samples were tested for microbiological analysis. We cannot discard the existence of false-negative CMT results that could affect the prevalence of MRSA IMI in the studied farm. In addition, we should take into account that this study focuses on a single herd and the true epidemiology of MRSA-ST398 in Brazilian dairy herds remains unknown.

None of the strains harboured any of the virulence genes studied, or any of the human-associated IEC genes, which is in line with former data on MRSA isolates of this lineage (Fluit 2012). In contrast, all isolates were multidrug resistant, including tetracycline resistance. Tetracycline resistance in MRSA ST398 has been reported as a consequence of the intensive use of this antimicrobial in livestock and seems to be endemic in MRSA of this lineage (Feßler *et al.* 2010; Gómez-Sanz *et al.* 2010; Fluit 2012; Lozano *et al.* 2012b).

One strain contained the gene *lnu*(B) and the recently characterized *lsa*(E), both of which confer resistance to lincosamides (Wendlandt *et al.* 2013a). Interestingly, this cluster also carried the recently characterized *spw* spectinomycin resistance gene (Wendlandt *et al.* 2013b). These three genes were clustered together and their genetic environment was identical to the one recently described in staphylococci among Spanish MRSA ST398 isolates (Lozano *et al.* 2012a), evidencing altogether the antimicrobial resistance acquisition capacities of geographically distinct MRSA ST398 isolates.

To our knowledge, this study is the first description of MRSA ST398 in animals in Brazil and also the first description in cow isolates in Latin America. A single former study has reported *Staph. aureus* CC398 in animals in Latin America in pigs in Peru (Arriola *et al.* 2011).

In conclusion, MRSA was isolated in 4% of investigated animals, corresponding to 11·1% of animals with mastitis coming from a single farm. All MRSA isolates belonged to the lineage ST398 were multidrug resistant and lacked important virulence genes. In Brazil, epidemiological studies of *Staph. aureus* in cattle are scarce, but are essential to gain knowledge on the circulating lineages responsible for cow mastitis in such milk producer country. Further surveillance on MRSA on animals should be conducted for a better understanding on the transmission routes of MRSA of different lineages among animals and humans.

Materials and methods

A total of 400 milk samples from 100 cows from one farm using milking technology (machine-milked cows) in the State of São Paulo (Brazil) was evaluated (one milk sample per mammary gland). The California mastitis test (CMT) was used for the diagnosis of clinical and subclinical mastitis (Schalm and Noorlander 1957). A single aseptic milk sample (20 ml) was collected from all CMT-positive quarters after udder preparation by the

Table 2 Oligonucleotides used for PCR detection of virulence genes

Gene	Primer	Size (pb)	Reference
lukF/lukS	F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAAGTGTATTGGATAGCAAAAGC	443	Jarraud *et al.* (2002)
tst	F: TTCACTATTTGTAAAAGTGTCAGACCCACT R: TACTAATGAATTTTTTTATCGTAAGCCCTT	180	Jarraud *et al.* (2002)
eta	F:ACTGTAGGAGCTAGTGCATTTGT R:TGGATACTTTTGTCTATCTTTTTCATCAAC	190	Jarraud *et al.* (2002)
etb	F: CAGATAAAGAGCTTTATACACACATTAC R: AGTGAACTTATCTTTCTATTGAAAAACACTC	612	Jarraud *et al.* (2002)
hlb	F: GTTGGTGCTCTTACTGACAA R: TGTGTACCGATAACGTGAAC	479	van Wamel *et al.* (2006)
scn	F: AGCACAAGCTTGCCAACATCG R: TTAATATTTACTTTTTAGTGC	258	van Wamel *et al.* (2006)
chp	F: TTTACTTTTGAACCGTTTCCTAC R: GTCCTGAATTCTTAGTATGCATATTCATTAG	366	van Wamel *et al.* (2006)
sak	F: AAGGCGATGACGCGAGTTAT R: GCGCTTGGATCTAATTCAAC	223	van Wamel *et al.* (2006)
sea	F: AGATCATTCGTGGTATAACG R: TTAACCGAAGGTTCTGTAGA	120	van Wamel *et al.* (2006)
sep	F: AATCATAACCAACCGAATCA R: TCATAATGGAAGTGCTATAA	500	van Wamel *et al.* (2006)

farm personnel. The milk samples were collected according to NMC procedures for collecting milk samples (http://www.nmconline.org/sampling.htm), prior to routine milk-out. Mastitis was considered when at least one of the 4 milk samples obtained from a cow was positive by CMT, and all CMT-positive milk samples were further studied for *Staph. aureus* recovery.

Milk samples of mastitic cows were plated on blood agar 5%, incubated at 37°C and readings were taken after 24, 48 and 72 h of incubation. Characteristic colonies were preliminary identified using Gram staining, catalase, coagulase and DNase tests. Susceptibility to oxacillin and cefoxitin was tested by the disc-diffusion agar method (EUCAST 2014). Molecular identification of *Staph. aureus* (*nuc* gene) and detection of the methicillin-resistant gene *mec*A was performed by a multiplex PCR (Gómez-Sanz *et al.* 2010).

All MRSA isolates were characterized by *agr*-allotype, *spa*-typing, determination of staphylococcal cassette chromosome *mec* (SCC*mec*) and multilocus sequence typing (MLST), by specific PCRs and subsequent sequencing (Shopsin *et al.* 2003; http://spaserver.ridom.de; IWG-SCC, 2009; www.mlst.net). In addition, pulsed-field-gel-electrophoresis (PFGE) of genomic DNA of MRSA strains, previous digestion with *Apa*I enzyme, was carried out applying the HARMONY protocol guidelines (Murchan *et al.* 2003) and with switching times of electrophoresis for *Apa*I digests as those implemented by Kadlec *et al.* (2009).

The presence of the genes encoding the Panton-Valentine-leukocidin (*lukF*/*lukS*), toxic-shock-syndrome-toxin (*tst*), and exfoliative-toxin A (*eta*) and B (*etb*) was analysed by PCR (Jarraud *et al.* 2002). The presence of the genes of the immune evasion cluster (IEC) was analysed as previously recommended (van Wamel *et al.* 2006) (Table 2).

Susceptibility testing to penicillin, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, trimethoprim-sulphametoxazole, ciprofloxacin and chloramphenicol was performed by the disc-diffusion agar method (EUCAST 2014). The presence of 16 antimicrobial resistance genes [*mec*A, *bla*Z, *tet*(K), *tet*(M), *tet*(L), *aac(6′)-aph(2″)*, *ant*4, *aadE*, *ant*3(9), *str*, *lnu*(A), *lnu*(B), *lnu(C)*, *vga*(A), *vga*(C) and *lsa*(E)] was investigated by specific PCRs (Gómez-Sanz *et al.* 2010; Lozano *et al.* 2012a; Wendlandt *et al.* 2013a,b), and in some cases also by sequencing [(*lnu*(B), *lsa*(E)]. The presence of amino acid changes in GyrA and GrlA proteins was investigated by PCR and sequencing (*gyr*A, *grl*A). In addition, mapping PCR was implemented to detect the antimicrobial resistance gene cluster *lnu*(B)-*lsa*(E)-*spw-aadE* (Lozano *et al.* 2012a; Wendlandt *et al.* 2013a,b). Positive and negative controls from the collection of the University of La Rioja were used in all PCRs.

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Conflict of Interest

No conflict of interest to be declared.

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