ORIGINAL ARTICLE

First Detection of Methicillin-Resistant *Staphylococcus aureus* ST398 and *Staphylococcus pseudintermedius* ST68 from Hospitalized Equines in Spain

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Impacts

- The first description of methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 in horses in Spain and one of the possibly earliest MRSA ST398 isolates in this country is reported. First detection of *Staphylococcus pseudintermedius* ST68 in horses.
- The identification of *dfrK* gene within the chromosomally located transposon Tn*559* in MRSA ST398 from horses is a novel observation.
- A *mec*A-positive *S. pseudintermedius* ST68 isolate showed a susceptible phenotype to oxacillin. This finding enhances the need for *mec*A gene testing in routine analysis to avoid treatment failures and for a better evaluation on anti-microbial resistance prevalence.

Keywords:
MRSA ST398; *mec*A-positive *S. pseudintermedius* ST68; Tn*559*; equidaes; Spain

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Summary

Eight coagulase-positive staphylococci from equines with different pathologies obtained between 2005 and 2011 were investigated. Isolates were characterized by different molecular techniques (*spa*-, *agr*-, MLST), and clonal relatedness of strains was investigated by ApaI and SmaI PFGE. Anti-microbial resistance and virulence profiles were determined. Six isolates were identified as *Staphylococcus aureus*, and two as *Staphylococcus pseudintermedius*. Of these, four isolates were methicillin-resistant *S. aureus* (MRSA) ST398 and one *S. pseudintermedius* was *mec*A positive and typed as ST68. One MRSA ST398 strain was isolated in 2005 and might be one of the earliest MRSA ST398 descriptions in Spain. All 5 *mec*A-positive strains were multidrug resistant and were isolated from hospitalized equines. Three MRSA ST398 strains carried the recently described transposon Tn*559* within the chromosomal *radC* gene. The *mec*A-positive *S. pseudintermedius* ST68 strain was also multidrug resistant and harboured the *erm*(B)-Tn*5405*-like element. This ST68 strain presented a clear susceptible phenotype to oxacillin and cefoxitin regardless of the presence of an integral and conserved *mec*A gene and *mecA* promoter, which enhances the need for testing the presence of this gene in routine analysis to avoid treatment failures. These data reflect the extended anti-microbial resistance gene acquisition capacities of both bacterial species and evidence their pathogenic properties. The first detection of MRSA ST398 and *S. pseudintermedius* ST68 in horses in Spain is reported.

Introduction

Staphylococcus aureus and *Staphylococcus pseudintermedius* are coagulase-positive staphylococci (CoPS) isolated from different animal species and commonly implicated in opportunistic infections (Ruscher et al., 2009; Couto et al., 2011; van Duijkeren et al., 2011a; Sieber et al., 2011; Pantosti, 2012; Gómez-Sanz et al., 2013). Methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) are emerging pathogens of different animal species, including humans (Ruscher et al., 2009; Kadlec et al., 2010; Perreten et al., 2010; Couto et al., 2011, 2012;

van Duijkeren et al., 2011a; Lozano et al., 2011a; Sieber et al., 2011; Pantosti, 2012). Pigs are the major host for MRSA of the multilocus sequence type (MLST) ST398 with a worldwide distribution, and cases of human infection due to MRSA of this genetic lineage are steadily increasing (Witte et al., 2007; Lozano et al., 2011a,b; Pantosti, 2012). MRSP is mainly isolated from dogs, with MRSP ST71 and ST68 as the predominant lineages detected in Europe and North America, respectively (Ruscher et al., 2009; Perreten et al., 2010; van Duijkeren et al., 2011a; Gómez-Sanz et al., 2011). In recent years, there has been an increase in the occurrence of MRSA ST398 in hospitalized horses in different countries, especially in Europe (Van den Eede et al., 2009; Haenni et al., 2010; van Duijkeren et al., 2011a; Sieber et al., 2011; Couto et al., 2012), and the first suspected horse-to-human transmission of MRSA ST398 causing a human infection has been recently described (van Duijkeren et al., 2011b). In contrast, *S. pseudintermedius* and MRSP have been only detected in equidaes at rare occasions (Ruscher et al., 2009; De Martino et al., 2010; Haenni et al., 2010), and even though cases of human infection have been detected, narrow host adaptability is expected for this bacterial species (van Duijkeren et al., 2011a).

Given that molecular characteristics of *S. aureus* and *S. pseudintermedius* in equidaes are poorly understood, and due to the lack of information on this subject in Spain, the objective of this study was to identify and characterize the CoPS isolated from clinical samples of hospitalized equines in Spain.

Materials and Methods

Origin of isolates and characteristics of animals

Between 2005 and 2011, 39 samples from hospitalized equines showing different pathologies were received and analysed in the Infectious Diseases Laboratory of the Veterinary Faculty of the University of Zaragoza (Spain). Eight potential CoPS (from 7 horses and one donkey) were recovered from these samples (Table 1). Most CoPS-positive samples came from infections related to the skin or mucosa. Six of the 8 animals (equines 1, 3, 4, 6–8) were under anti-microbial treatment previous to sampling: five of them received penicillin plus gentamicin and the other one trimethoprim/sulphamethoxazole (horse 7).

Bacterial identification

Isolates were identified by conventional methods (Gram-staining, catalase and oxidase test and DNase production). Identification of *S. aureus* and *S. pseudintermedius* was confirmed by a multiplex PCR that amplifies the *nuc* gene of *S. aureus* or *S. (pseud)intermedius* (Baron et al., 2004). Discrimination between *S. intermedius* and *S. pseudintermedius* was made by restriction fragment length polymorphism of the *pta* gene with MboI enzyme (Bannoehr et al., 2009). Presence of the *mec*A gene was investigated by PCR in all isolates (CRL-AR, 2009).

Molecular typing of isolates

All *S. aureus* isolates were subjected to *spa* typing as previously described (http://www.spaserver.ridom.de), and sequences were analysed using Ridom Staph-Type software version 2.0.21 (Ridom GmbH). The *agr* types were investigated by specific PCRs (Shopsin et al., 2003)). Multilocus sequence typing (MLST) on *S. aureus* was performed as recommended (http://www.mlst.net). SCC*mec* types I to V were investigated in *S. aureus* isolates by PCR of the *ccr* recombinases (1 to 5) and the *mec* gene complex type (A to C) as recommended by IWG-SCC (IWG-SCC, 2009; Kondo et al., 2007). An additional PCR was performed to differentiate different subtypes of SCC*mec* IV (a to d) (Zhang et al., 2005).

Staphylococcus pseudintermedius isolates were investigated by species-specific *spa* typing (Moodley et al., 2009). MLST on this bacterial species was performed on five housekeeping genes (*pta*, *cpn60*, *tuf*, 16S rDNA and *agrD*) followed by sequence comparison of the alleles with those deposited in GenBank/EMBL databases (Bannoehr et al., 2007). *S. pseudintermedius* isolates were tested for the presence of the SCC*mec* types I to V, in addition to the so far *S. pseudintermedius*-specific SCC*mec* V_T and II–III (Perreten et al., 2010).

Anti-microbial resistance profile

Susceptibility testing and interpretation was made as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012), except for streptomycin and fusidic acid, for which methods and breakpoints recommended by the Société Française de Microbiologie were used (SFM, 2010). Anti-microbials tested were as follows (μg/disc): penicillin (10U), oxacillin (1), cefoxitin (30) erythromycin (15), clindamycin (2), gentamicin (10), kanamycin (30), streptomycin (10U), tobramycin (10), tetracycline (30), trimethoprim/sulphamethoxazole (1.25 + 23.75), trimethoprim (5), chloramphenicol (30), ciprofloxacin (5), mupirocin (200), fusidic acid (10), vancomycin (30) and linezolid (30) (CLSI, 2012).

The double-disc diffusion test (D-test) was performed to detect inducible clindamycin resistance (CLSI, 2012). Minimum inhibitory concentration (MIC) to oxacillin and cefoxitin was determined by agar dilution method on three independent assays using *S. aureus* ATCC29213

Table 1. Characteristics of the *S. aureus* and *S. pseudintermedius* isolates obtained from animals tested

Characteristics of the isolates							Characteristics of the animals			
Strain	year	Type[a]	*spa-agr*-MLST-SCC*mec*	Resistance phenotype[b]	Resistance genes detected	Virulence genes detected	Animal ID	Age (years)	Breed	Clinical disease
C3865	2005	MRSA	t011-I-ST398-IV[a]	Pen-Oxa-Fox-Tet-Gen-Tob	*blaZ*, *mec*A, *tet*(K), *tet*(M), *aacA-aphD*	*cna, hla, hlb, hld, hlg*	Horse-1	5	Spanish Purebred	Mumps
C4667	2010	MRSA	t011-I-ST398-IV[a]	Pen-Oxa-Fox-Tet-Gen-Tob-Str-Tmp	*blaZ*, *mec*A, *tet*(K), *tet*(M), *aacA-aphD*, *str*, *dfr*(G), *dfrK*[c]	*cna, hla, hlb, hld, hlg*	Horse-2	4	Lusitano	Hardening of salivary gland
C4668	2011	MRSA	t011-I-ST398-IV[a]	Pen-Oxa-Fox-Tet-Gen-Tob-Tmp	*blaZ*, *mec*A, *tet*(K), *tet*(M), *a acA-aphD*, *dfr*(G), *dfrK*[c]	*cna, hla, hlb, hld, hlg*	Horse-3	9	KWPN[d]	Post-surgical injury (colic)
C4670	2011	MRSA	t011-I-ST398-IV[a]	Pen-Oxa-Fox-Tet-Gen-Tob-Tmp	*blaZ*, *mec*A, *tet*(K), *tet*(M), *aacA-aphD*, *dfrK*[c]	*cna, hla, hlb, hld, hlg*	Horse-4	12.5	Spanish Purebred	Post-surgical injury (colic)
C5343	2009	MSSA	t10576-II-ST1660	Pen	*blaZ*	*hla, hlb, hld, hlgv, cna, sem, sei*	Horse-5	8	Spanish Purebred	Arthritis
C5611	2010	MSSA	t1508-III-ST1	Pen	*blaZ*	*lukED, hlb, hld, hlgv, cna, seh*	Horse-6	7	Mixed Shire bred	Abscess axilla
C5337	2008	*mec*A- + SP	t06-IV-ST68-V_T	Pen-Tet-Ery-Cli[e]-Kan-Str-Sxt-Cip[f]	*blaZ*, *mec*A[g], *tet*(K), *tet*(M), *aacA-aphD*, [*erm*(B)-*aadE-sat4-aphA3*][h], *dfr*(G)	*lukS/F-I*, *siet*, *se-int*	Horse-7	2.5	Spanish Purebred	Suppurative bone sequestration
C5348	2009	MSSP	NT[i]-II-ST184	Pen-Tet	*blaZ*, *tet*(K), *tet*(M)	*lukS/F-I*, *siet*, *se-int*	Donkey-1	6	Spanish donkey	Emphysema of skin and injury

[a]MRSA, methicillin-resistant *Staphylococcus aureus*; *mec*A+ SP, *mec*A-positive *S. pseudintermedius*; MSSA, methicillin-susceptible *S. aureus*; MSSP, methicillin-susceptible *S. pseudintermedius*.
[b]Pen, penicillin; Oxa, oxacillin; Fox, cefoxitin; Tet, tetracycline; Ery, erythromycin; Cli, clindamycin; Gen, gentamicin; Tob, tobramycin; Kan, kanamycin; Str, streptomycin; Sxt, trimethoprim/sulphamethoxazole; Cip, ciprofloxacin; Tmp, trimethoprim.
[c]The *dfrK* gene was part of the transposon Tn*559*, which was inserted in the chromosomal *radC* gene.
[d]Koninklijk Warmbloed Paardenstamboek Nederland.
[e]The double-disc diffusion test (D-test) revealed a constitutive resistance phenotype to clindamycin.
[f]Amino acid substitutions in Ser80Ile of GrlA protein and Ser84Leu in GyrA were detected.
[g]The gene *mec*A is present but there is a susceptible phenotype to oxacillin and cefoxitin.
[h]Resistance genes physically linked.
[i]Non-typeable.

and *Enterococcus faecalis* ATCC29212 as reference strains and quality controls (CLSI, 2012). Presence of the following anti-microbial resistance genes was analysed by PCR in all isolates: *mec*A, *blaZ*, *tet*(K), *tet*(M), *tet*(L), *erm*(A), *erm*(B), *erm*(C), *erm*(T), *aacA-aphD*, *aphA-3*, *aadD*, *aadE*, *aadA*, *str*, *sat4*, *dfr*(A), *dfr*(D), *dfr*(G) and *dfrK* (Gómez-Sanz et al., 2010, 2011).

The presence of the *erm*(B)-Tn*5405*-like element, which carries the macrolides–lincosamides–streptogramines B resistance gene *erm*(B), streptomycin and kanamycin/neomycin resistance determinants (*aadE* and *aphA3*, respectively), in addition to the streptothricin resistance gene *sat4*, was investigated by mapping PCRs (Gómez-Sanz et al., 2011). Mutations in the quinolone resistance–determining region (QRDR) of GyrA and GrlA proteins, in addition to relevant mutations outside QRDR of GyrA, were investigated in the ciprofloxacin-resistant isolate (Gómez-Sanz et al., 2011).

Determination of *mecA* and *blaZ* regulatory elements

Presence of the regulatory genes of *mec* [*mec*I (*mec*A transcription repressor) and *mec*R1 (sensor/signal transducer)] and *bla* (*bla*I/*bla*R1) operons was investigated by PCR in all isolates (Black et al., 2011; Gómez-Sanz et al., 2011).

In addition, primers mecA(SP)-ups (5′-TGGAATTAAC GTGGAGACGA-3′) and mecA(SP)-rv (5′-TTATTCATC TATATCGTATT-3′) (GenBank accession no. AM904732) were used to amplify the entire *mec*A gene in strain C5337. The *mec*A promoter region of this strain was also characterized by PCR and sequencing (Gómez-Sanz et al., 2011).

Location and genetic environment of the *dfrK* gene in *Staphylococcus aureus* isolates

Pulsed field gel electrophoresis (PFGE) after linearization of plasmid DNA with S1 (Takara) and digestion of genomic DNA with ICeuI (New England Biolabs) was performed as previously described on *dfrK*-positive isolates (López et al., 2012). S1-PFGE and I-CeuI-PFGE gels obtained were subjected to hybridization and detection with specific probes of the *dfrK* to determine the chromosomal DNA and/or plasmid location of this gene in the genome (López et al., 2012). The possibility that the *dfrK* gene formed part of the recently described Tn*559*, which carries the transposase genes *tnpA*, *tnpB* and *tnpC*, in addition to the *dfrK* gene, was investigated by specific PCRs of the transposase genes and *dfrK* in different combinations (Fig. 1, Table 2). Given that this transposon has been so far located integrated in the chromosomal *radC* gene (Kadlec and Schwarz, 2010; Fessler et al., 2011; López et al., 2012), a specific PCR was employed to evaluate the possible integration of Tn*559* within *radC* (Table 2), with two possible amplicon sizes achieved: (i) 114 bp when *radC* is intact and (ii) 4,403 bp if Tn*559* is located within the *radC* gene. An additional PCR using dfrK-fw and radC_SA-rv was employed for confirmation of the integration of the Tn*559* within the *radC*.

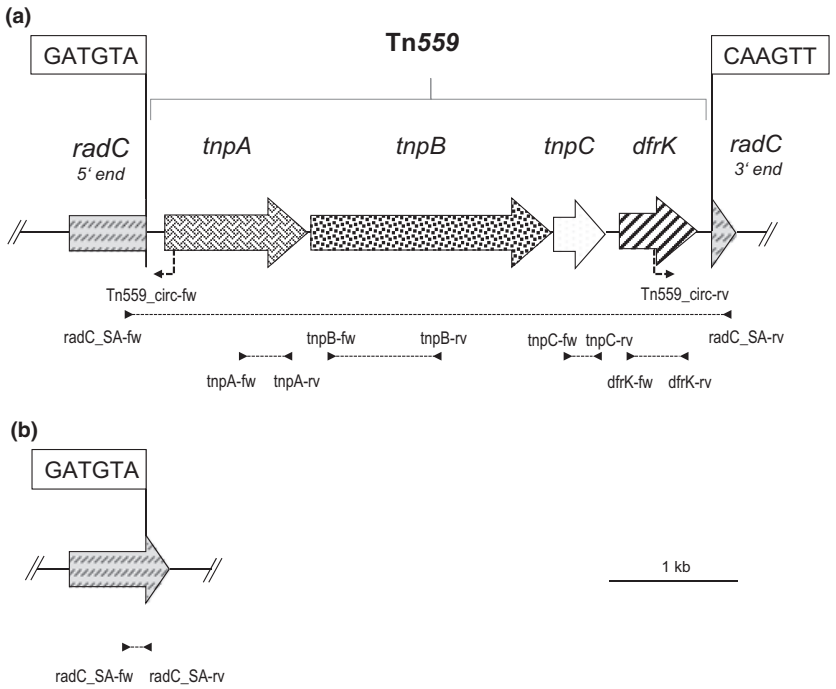

Fig. 1. Schematic representation of Tn*559* and its chromosomal integration region within the chromosome of *S. aureus*. The arrows indicate the size and direction of transcription of the transposase (*tnpA*, *tnpB*, *tnpC*) and trimethoprim resistance (*dfrK*) genes as well as the gene coding a DNA repair protein (*radC*) that serves as integration site. Recognition site (5′-GATGTA-3′) is boxed. A size in kb scale is given. (a) Transposon Tn*559* integrated within the chromosomal *radC* of *S. aureus* and primers employed for its detection and potential functionality. (b) Chromosomal segment of *S. aureus* carrying an integral copy of the *radC* gene.

Table 2. Primers employed in this study to investigate the presence of the conserved *dfrK*-carrying transposon Tn*559* and PCR conditions used

Gene or region amplified	Primer	Primer sequence (5′→3′)	Amplicon (bp)	Reference (GenBank/EMBL accession no.)
dfrK	dfrK-fw	GAGAATCCCAGAGGATTGGG	423	Gómez-Sanz et al., (2010)
	dfrK-rv	CAAGAAGCTTTTCGCTCATAAA		
tnpA	tnpA-fw	GCCACTTGGGACAATCAAAT	328	This study (FN677369)
	tnpA-rv	CAACATCCCATCCTTCCCTA		
tnpB	tnpB-fw	AAATCGTTGGGTTCGTTTTG	826	This study (FN677369)
	tnpB-rv	AAAGCGAGGTTTTGCTCTTG		
tnpC	tnpC-fw	GGCGAAGCAGAAATCACAA	235	This study (FN677369)
	tnpC-rv	AAGGATTTCCTCCGAACGAG		
radC	radC_SA-fw	CGGTGAGAGAAAATGCCAAT	114/4,403[a]	This study (FN677369)
	radC_SA-rv	TCAAACCACACTCCTTCAACC		
Tn*559*-circ	Tn559_circ-fw	TCCATGAACTCGTACAGCAA	778	Kadlec and Schwarz (2010)
	Tn559_circ-rv	TGGTTGTGAAATTGTCCATTC		

The PCRs were performed using BioTaq™ DNA Polymerase (Bioline) and the conditions were as follows: initial cycle of 3 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, with a final step of 10 min at 72°C. An additional PCR was performed for *radC* gene using LA Taq DNA polymerase (Takara) and following manufacturer recommendations.

[a]Amplicon size only obtained when Tn*559* is inserted within *radC* gene.

Virulence genes profile

The presence of the leukocidin genes *lukS/F-PV*, *lukM* and *lukE/D*, the haemolysin genes *hla*, *hlb*, *hld*, *hlg* and *hlgv* and the exfoliative genes *eta*, *etb* and *etd* was determined in all isolates by specific PCRs (Lina et al., 1999; Jarraud et al., 2002). The toxic shock syndrome toxin *tst* and the collagen adhesion precursor *cna* genes were likewise investigated (Jarraud et al., 2002; Witte et al., 2007). PCR-based determination of 18 enterotoxin genes was performed on all isolates (Hwang et al., 2007).

In addition, the leukocidin *lukS/F-I*, the exfoliatin genes *siet, expA* and *expB* and the enterotoxin genes *se-int* and sec_{canine} were investigated by PCR in *S. pseudintermedius* isolates (Gómez-Sanz et al., 2013).

Clonal relatedness of strains

Pulsed field gel electrophoresis after the digestion of genomic DNA with SmaI or ApaI macrorestriction enzymes was performed on *S. aureus* and *S. pseudintermedius* isolates (Gómez-Sanz et al., 2010). PFGE of ApaI- or SmaI-digested plugs was run for 20 h at 6V/cm using pulsed time ramping from 2 to 5 s.

Results

Identification of isolates and molecular typing

Six of the 8 isolates were identified as *S. aureus* (Table 1). Four *S. aureus* were MRSA and typed as t011(*spa*)-I(*agr*)-ST398(MLST)-SCC*mec*IVa. The remaining two *S. aureus* were MSSA; one showed a novel *spa* type registered as t10576 (r04-r20-r69-r31-r70-r13-r16-r16-r16) and presented ST1660. The other MSSA was t1508-ST1.

The remaining two isolates were *S. pseudintermedius*. One isolate was *mec*A positive and typed as t06-IV-ST68-SCC*mec*V_T (5C2&5). The other isolate, recovered from a donkey, was a MSSP, *spa* non-typeable and ST184.

Characteristics of β-lactam resistance and presence of regulatory elements

In addition to MRSA isolates, both MSSA isolates were penicillin resistant (harbouring the *blaZ* gene) and all amplified the *bla*I/*bla*R regulatory elements. The four MRSA ST398 isolates showed oxacillin MIC values of 16–32 μg/mL, while cefoxitin MICs were 16 μg/mL. Both MSSA isolates exhibited oxacillin and cefoxitin MIC values of 0.25–0.5 μg/mL and 2 μg/mL, respectively. The four MRSA ST398 isolates lacked the transcription repressor *mec*I gene and showed a truncated *mecR*1 gene, as expected for the presence of the SCC*mec* IVa.

Both *S. pseudintermedius* isolates were penicillin resistant harbouring the *blaZ* gene and both regulatory elements (*bla*I/*bla*R). Disc diffusion method showed that both strains were susceptible to oxacillin (inhibition zone: 21–22 mm) and cefoxitin (35–40 mm), even though one of them was *mec*A positive (C5337). Macrodilution assays confirmed that both isolates were oxacillin (MIC = 0.25 μg/mL) and cefoxitin (MIC = 0.25–0.5 μg/mL) susceptible. The *mec*A-positive *S. pseudintermedius* strain C5337 also lacked the *mec*I gene and showed a truncated *mec*R, as expected for the presence of the SCC*mec*V_T element.

Sequencing of the complete *mec*A gene of *S. pseudintermedius* strain C5337 and its promoter region revealed no mutations that might explain such atypical phenotype. Sequence comparison analysis of the complete *mec*A of C5337 with deposited sequences on the GenBank database

evidenced complete identity to *mec*A found within the SCC*mec*V_T of a MRSP ST68 strain (GenBank accession no. FJ544922) among others.

Anti-microbial resistance profile to non β-lactams

Complete anti-microbial resistance phenotype and genotype of strains is shown in Table 1. The four MRSA isolates were multidrug resistant (MDR) (resistance to at least three classes of anti-microbial agents). They presented the tetracycline resistance genes *tet*(K) and *tet*(M) and the aminoglycosides resistance gene *aacA-aphD*, while three of them were also trimethoprim resistant [*dfrK* and/or *dfr*(G)] and one showed additional resistance to streptomycin (*str*) (Table 1).

As for *S. pseudintermedius*, the multidrug resistance gene cluster *aadE-sat4-aphA3* and its physical linkage to *erm*(B) gene [*erm*(B)-Tn*5405*-like element] was evidenced in the *mec*A-positive ST68 strain. This isolate showed resistance to tetracyclines, aminoglycosides, macrolides–lincosamides and trimethoprim (Table 1). The MSSP isolate was tetracycline resistant and presented both *tet*(K) and *tet*(M) genes.

Genetic environment of the dfrK *gene*

The three MRSA ST398 strains that carried the *dfrK* gene harboured a complete and conserved transposon Tn*559* (Fig. 1). I-CeuI-PFGE hybridization experiments revealed that the *dfrK* gene was only located within the chromosomal DNA. In addition, based on the PCR amplicon obtained, Tn*559* was located within the chromosomal *radC* gene. Circular forms were also detected indicating a functional activity.

Toxin gene pattern

All MRSA ST398 strains were positive for the *cna* and some haemolysin genes, whereas both MSSA strains showed a more extensive virulence profile; the leukocidin gene *lukED* was detected in MSSA ST1 (Table 1).

The leukocidin *lukS/F-I*, exfoliatin *siet* and enterotoxin *se-int* genes were present in both *S. pseudintermedius* strains.

Clonal relatedness of strains investigated

SmaI PFGE showed a slightly higher discriminative power for the differentiation of both MSSA isolates with the implemented PFGE conditions (Fig. 2). As expected, MRSA ST398 strains were not digested by the SmaI enzyme. The four MRSA ST398 isolates presented closely related patterns by ApaI-PFGE regardless they were isolated in different years from unrelated horses (Fig. 2).

Both enzymes were useful to discriminate both *S. pseudintermedius* isolates, ApaI exhibiting more restriction sites.

Discussion

Even though MRSA ST398 has become the predominant MRSA lineage in diseased horses in Europe in the last years (Sieber et al., 2011), this is the first report on MRSA in

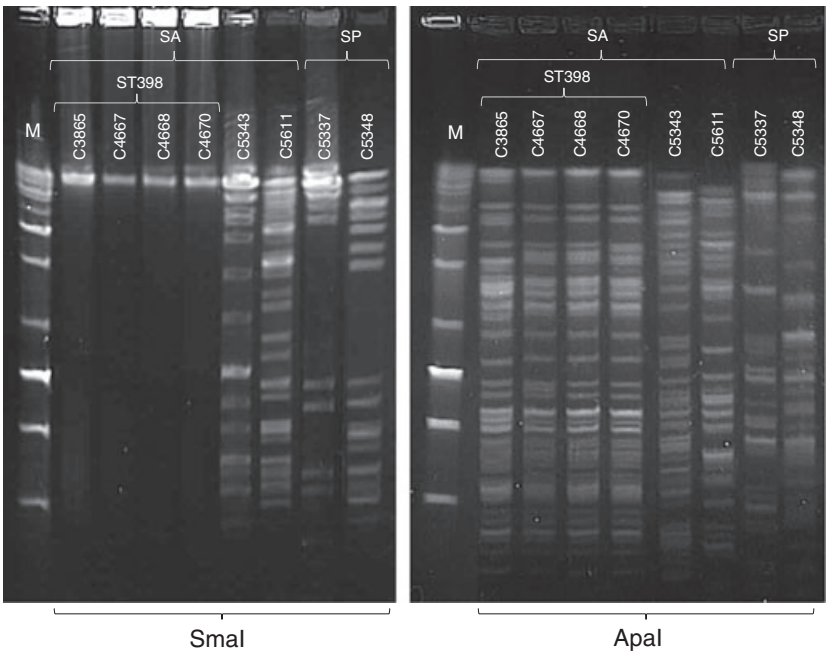

Fig 2. Pulsed Field Gel Electrophoresis (PFGE) pattern of chromosomal DNA digested with SmaI and ApaI macrorestriction enzyme of all *S. aureus* (SA) and *S. pseudintermedius* (SP) strains investigated. Strains C3865, C4667, C4668 and C4670 (methicillin-resistant SA ST398); C5343 (methicillin-susceptible SA ST1660); C5611 (methicillin-susceptible SA ST1); C5337 (*mec*A-positive SP ST68); C5348 (methicillin-susceptible SP ST184). M, Low Range Pulse Marker (New England Biolabs).

equidaes in Spain. The first description of MRSA ST398 in Spain was reported in food samples of pig origin in 2008 (Lozano et al., 2009), and several reports have been later described on swine and people in contact with these animals (Gómez-Sanz et al., 2010; Lozano et al., 2011a,b). Our strain recovered in 2005 represents therefore the earliest case of MRSA ST398 in Spain to date and evidences its early presence in non-porcine animal species. The horse carrying this strain came from a horse farm located very close to animal husbandries and to a slaughterhouse. Unfortunately, potential risk factors for MRSA ST398 acquisition and infection could not be established in the rest of positive animals, given that these data were not collected at sampling. One of the MSSA belonged to ST1660, a so far uncommon lineage that was described for the first time in a borderline-oxacillin-resistant *S. aureus* strain from a diseased horse in Switzerland (Sieber et al., 2011). In contrast, isolates of the lineage ST1, which have been frequently detected before, appear highly adapted to different animal species (Pantosti, 2012).

ST68 is the predominant MRSP lineage in North America in dogs. In Europe, MRSP of this lineage have only been detected in Portuguese dogs (Couto et al., 2011). To our knowledge, this is the first description of *S. pseudintermedius* ST68 in horses and suggests a less restricted geographic and animal host spectrum than expected. On the other hand, an expanded recently described MLST approach, which includes four additional loci, has allowed the discrimination of three sequence types (ST29, ST30 and ST68) within a group of MRSP ST68 isolates (Solyman et al., 2013), showing a lower clonal genetic background than believed. Background of the patient could not be determined, and therefore, the role of possible dog contact cannot be ruled out. Because very scarce data are so far available on the nasal or skin microbiota of donkey (Ruscher et al., 2009), the presence of *S. pseudintermedius* in one animal is remarkable. Curiously, ST184 was recently described in a MSSP of dog origin in Spain (Gómez-Sanz et al., 2012).

Although the epidemiological significance of the present data is very low due to the limited number of samples, the proportion of *mec*A-positive isolates (5/8, 62.5%) is notable. Interestingly, *mec*A-positive *S. pseudintermedius* ST68 strain C5337 was susceptible to both oxacillin and cefoxitin by both disc diffusion and agar dilution methods. Several reports have indicated the low reliability of cefoxitin disc diffusion tests to determine the methicillin resistance phenotype of *S. pseudintermedius* using conventional guideline breakpoints [≤21 mm resistant, ≥22 mm susceptible (CLSI, 2012)] (Bemis et al., 2012). Also, that study has suggested an epidemiological breakpoint for cefoxitin zone diameter of ≤30 mm for resistant and ≥31 mm for susceptible, after analysing a large *S. pseudintermedius* collection (Bemis et al., 2012). Remarkably, strain C5337 remained not only susceptible to cefoxitin following the latter criteria, but also to oxacillin, which is still considered a reliable marker of methicillin resistance in this species. The fact that neither the *mec*A gene nor its promoter region presented mutations that could explain this atypical phenotype is surprising. The detection of the entire *bla*I/*bla*R1 regulatory elements may be speculated to be implicated in this stringent regulation of *mec*A expression. Moreover, other factors (different mutations on surface proteins, lower permeability, etc.) or additional regulatory elements might affect the *mec*A expression levels in C5337 (McCallum et al., 2010), which warrants further in-depth investigations.

It is interesting to remark that all strains resistant to penicillin, gentamicin or trimethoprim/sulphamethoxazole were recovered from animals that had received previous treatment with those anti-microbials. The complete *erm*(B)-Tn*5405*-like resistance gene cluster or variants seem to be ubiquitous on MRSP strains (Perreten et al., 2010; van Duijkeren et al., 2011a; Gómez-Sanz et al., 2011); this enhances its MDR pattern and increases concerns when addressing the possible risk of zoonotic transmission.

The recently described *dfrK*-carrying transposon Tn*559* has been detected so far in a porcine isolate and some poultry/poultry products in staphylococci, all of the lineage ST398 (Fessler et al., 2011; Kadlec et al., 2012). Hence, the detection of the Tn*559* in MRSA ST398 from horses is a novel observation and reflects the anti-microbial resistance gene acquisition capacity of MRSA ST398 isolates of different origins. Primary target site for the integration of Tn*559* is designated *att559* and is located within the chromosomal *radC* gene (Kadlec et al., 2010). This evidences a preferable chromosomal location within the bacterial genome. Circular forms were also detected, indicating a functional activity and therefore the possibility of exchange between bacteria.

The low occurrence of virulence genes in *S. aureus* isolates that belong to ST398 is in agreement with previous reports (Gómez-Sanz et al., 2010; Lozano et al., 2011a,b). By contrast, although very scarce data are available on the occurrence of virulence genes in *S. pseudintermedius* isolates, a few reports have shown ubiquity of the same set of virulence determinants: the leukocidin *lukS/F-I*, the exfoliatin *siet* and enterotoxin *se-int* genes, between MSSP and MRSP of different lineages (van Duijkeren et al., 2011a; Gómez-Sanz et al., 2011, 2013; Ben Zakour et al., 2012).

Our MRSA ST398 strains showed closely related PFGE patterns, although they were isolated in three different years from unrelated horses. These results suggest a clonal spread of MRSA ST398 in these animals and ensure further epidemiological investigations. Low discriminatory power of ApaI enzyme might also be speculated. Although

contamination at the clinic should be negligible, this option cannot be discarded.

The first description of MRSA ST398 in horses in Spain and one of the possibly earliest MRSA ST398 isolates in this country is reported. This is the first report on *S. pseudintermedius* ST68 in horses and the first detection of this American associated lineage in Europe in a non-canine animal species. The detection of the Tn*559* in MRSA ST398 from horses is a novel observation and reflects its high antimicrobial resistance gene acquisition ability. The finding of a *mec*A-positive *S. pseudintermedius* strain that shows susceptibility to oxacillin and cefoxitin enhances the need for testing the presence of the *mec*A gene in routine analysis to avoid treatment failures. Given that all *mec*A-positive strains were MDR, treatment options are compromised. Due to the close contact between horses and owners, potential public health implications on humans in contact with these animals are conceivable.

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