FULL PAPER *Bacteriology*

Isolation and Molecular Characterization of Methicillin-Resistant Staphylococci from Horses, Personnel and Environmental Sites at an Equine Hospital in Turkey

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ABSTRACT. The present study was carried out to assess the frequency of methicillin-resistant staphylococci (MRS) among racehorses (n=209) and veterinary personnel (n=13) as well as environmental surfaces (n=14) at an equine hospital in Adana, Turkey. In addition, species distribution, antimicrobial susceptibility, resistance genes, staphylococcal chromosomal cassette *mec* (SCC*mec*) type and clonality of these isolates were also investigated. MRS were identified by 16S rRNA sequencing, and typed by pulsed-field gel electrophoresis (PFGE). As a result, MRS was isolated in horses (48.3%), clinic staff (92.3%) and environmental samples (71.4%). Of the 123 MRS isolates, 118 isolates were identified as *Staphylococcus lentus*, and the remaining ones were found to be *S. sciuri* (n=3), *S. intermedius* (n=1) and *S. fleuretti* (n=1). All isolates were found to be susceptible against vancomycin, quinupristin-dalfopristin and rifampicin. Additionally, single or various combinations of resistance genes were detected among MRS isolates. SCC*mec* type II was identified in all isolates. Similar PFGE patterns were observed among MRS isolated from horses, humans, and environmental samples. Since MRS were concurrently isolated from horses and humans it is suggested that cross-transmission of MRS between horses and humans might occur. However, it cannot be ruled out that transmission is human to animal or animal to human.
KEY WORDS: antimicrobial resistance, horse, methicillin resistant staphylococci, molecular typing.

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Staphylococci are a diverse group of commensal organisms that colonize on the skin and mucous membranes of animals and humans. Some species have been recognized as opportunistic pathogens causing hospital-associated and community-associated infections in humans [18, 25]. The importance of coagulase-negative staphylococci (CNS) in veterinary medicine is increasing, and some CNS species have been implicated in different clinical cases of various animal species [24, 25, 30].

Methicillin resistance among staphylococci, in particular methicillin-resistant *Staphylococcus aureus* (MRSA), is an emerging problem and has become a human and veterinary medicine concern worldwide [8]. Methicillin resistance is mediated by a 78 kDa penicillin binding protein 2a (PBP2a) with a low affinity for beta-lactam antibiotics, which is encoded by the *mec*A gene located on a large mobile genetic element designated staphylococcal cassette chromosome *mec* (SCC*mec*) [14]. In addition, MRSA strains are not only resistant to beta-lactams but also frequently resistant to other classes of antimicrobial agents such as aminoglycosides and macrolides, and eleven different types of SCC*mec* have been reported so far (http://www.sccmec.org/Pages/SCC_TypesEN.html).

It has been reported that MRSA has been frequently isolated from unhealthy horses, transmitted between animals and humans [1, 33, 36, 37]. A high prevalence of methicillin-resistant staphylococci (MRS) colonization has also been reported in healthy horses in Europe [2, 4, 9, 20, 34] and Japan [38, 39].

Currently, there are no studies that have aimed to determine the presence of MRS in horses, humans and clinic environments, and molecularly characterize these agents in Turkey. The aims of this study were to investigate the occurrence of MRS in horses, clinic staff and clinic environments, (ii) to identify MRS strains at the species level by 16S rRNA sequencing, (iii) to investigate the SCC*mec* type, (iv) to determine whether methicillin-resistant coagulase-negative staphylococci (MRCNS) clones are shared by horses and clinic personnel including veterinarians and veterinary technicians by pulsed-field gel electrophoresis (PFGE) and (v) to determine antimicrobial susceptibility patterns and resistance genes of isolates.

MATERIALS AND METHODS

Sampling: A total of 209 equine patients admitted to an equine hospital in Adana, Turkey, for various medical problems and 13 clinic staff were sampled between December 2008 and March 2009. None of the animals and staff who had direct or indirect contact with horses had a known

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MRSA infection. Cotton swabs were taken from each nostril, and stored in Stuart's medium until laboratory analysis. In addition, samples were also collected from 14 environmental surfaces including door handles (n=4), restraining boxes (n=2), inspection equipments (n=3), X-ray devices (n=2), operating tables (n=2) and a stretcher (n=1). Ethical consent was obtained from the Mustafa Kemal University Medical Faculty Ethics Committee and Animal Ethical Committee.

Isolation and identification: The swabs were selectively enriched in tryptone soy broth (TSB) containing 7% sodium chloride and 2 μg/m*l* oxacillin and incubated at 35°C for 24 hr. Subsequently, the inoculum was spread onto mannitol salt agar containing 2 μg/m*l* oxacillin and incubated 35°C for 24–48 hr. A single presumptive staphylococcal colony was selected and identified phenotypically on the genus level by conventional biochemical tests. Methicillin resistance was phenotypically determined using oxacillin (1 μg) and cefoxitin (30 μg) by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) [6] and tested by PCR for the *mec*A gene. Furthermore, these strains were identified by sequencing the 16S rRNA gene using universal primers 16S20, 5'-AGA GTT TGA TCC TGG CTC AG-3', and 16S1390, 5'-GAC GGG CGG TGT GTA CAA-3' [27, 29]. Nucleotide sequences were compared with the published sequences in the National Center of Biotechnology Information databases (available online at http://www.ncbi.nlm.nih.gov), and 97% or higher similarity to a type strain was considered the criterion for establishing species identity. For reproducibility of the assay and comparative purposes, methicillin-resistant *S. aureus* ATCC 43300, *S. aureus* N315 (SCC*mec* type II) and methicillin-sensitive *S. aureus* ATCC 25923 strains were included in the test as positive controls.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing of MRS was performed according to a procedure outlined by the CLSI [6, 7]. The antimicrobial discs used in this study were erythromycin (15 μg), trimethoprim-sulfamethoxazole (1,25 μg/23,75μg), vancomycin (30 μg), gentamicin (10 μg), quinupristin-dalfopristin (15 μg), ciprofloxacin (5 μg), mupirocin (5 μg), fusidic acid (10 μg) rifampicin (5 μg), amoxicillin-clavulanic acid (20 μg/10 μg), clindamycin (2 μg) and tetracycline (30 μg). Since standardized CLSI breakpoints for mupirocin and fusidic acid are not available, the results for these antimicrobials were interpreted as previously reported [10, 32].

DNA extraction: DNA extraction was performed using a commercial genomic DNA extraction kit according to the manufacturer's instructions (InstaGene Matrix, Bio-Rad, Munich, Germany).

Detection of resistance genes: Antibiotic resistance genes associated with macrolides (*erm*A, *erm*B, *erm*C, *msr*A, *mph*C), lincosamides (*lun*A), aminoglycosides [*aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*], tetracyclines (*tet*K, *tet*M), mupirocin (*ileS-2*) and fusidic acid (*fus*B, *fus*C) were screened as previously reported using PCR [5, 13, 15–17, 19, 22, 28].

SCCmec typing: Staphylococcal cassette chromosome *mec* types of the isolates were determined using methods and primers described by Oliveira and de Lencastre [23].

PFGE analysis: PFGE was performed according to Mulvey *et al*. [21], and interpretation of results was performed according to Tenover *et al*. [31].

RESULTS

Detection of MRS: MRS were isolated from 101 (48.3%) horses, 12 (92.3%) clinic staff and 10 (71.4%) environmental samples. The results of 16S rRNA sequencing revealed the presence of four species: *S. lentus* (n=118), *S. sciuri* (n=3), *S. intermedius* (n=1) and *S. fleuretti* (n=1). *S. lentus* was the only MRS species isolated from clinic staff and environmental samples (Table 1).

Antimicrobial susceptibility testing: All MRS strains were sensitive to vancomycin, quinupristin-dalfopristin and rifampicin. Among the equine MRS isolates, variable rates of resistance were determined against other antibiotics including tetracycline (65.3%), clindamycin (18.8%), erythromycin (6.9%), gentamicin (5%), trimethoprim-sulfamethoxazole (3.0%), amoxicillin-clavulanic acid (2%), ciprofloxacin (2%), fusidic acid (2%) and mupirocin (1%) (Table 2). Tetracycline and clindamycin resistance were the most common, followed by gentamicin (16.7%), among MRCNS strains isolated from clinic staff. The MRCNS strains isolated from environmental sites showed higher resistance to tetracycline (90%), followed by gentamicin (10%), clindamycin (10%) and ciprofloxacin (10%) (Table 2).

PCR detection of antimicrobial resistance genes: Of the 83 tetracycline-resistant isolates, 73 possessed *tet*K and 4 had *tet*M, while 6 had both *tet*K and *tet*M. Among the erythromycin-resistant isolates (n=7), *mph*C was detected in all isolates, *erm*A and *mph*C were detected in 2 isolates, *mph*C was detected in 2 isolates, *erm*B, *erm*C and *mph*C were detected in one isolate, *msr*A and *mph*C were detected in one isolate, *erm*A, *msr*A and *mphC* were detected in one isolate. All gentamicin-resistant isolates carried only *aac(6')-aph(2")*. While the *ileS-2* gene was not detected in the mupirocin-resistant isolate, the *fus*B gene was detected in two fusidic acid-resistant isolates.

PFGE typing and SCCmec type: PFGE analysis revealed the presence of three pulsotypes (PTs) (A1-A2, B, C) among *S. lentus* isolates (Fig. 1). The dominant PT-A1 was isolated from 42 horses, all clinic staff and nine environmental sites. PT-A2, differing from PT-A1 by 2 band changes, was only isolated from 45 horses. PT-B was shared by four horses and one environmental site. PT-C was isolated from 5 horses only. The three *S. sciuri* strains isolated from horses displayed two pulsotypes (PT-A, PT-B), with two isolates displaying PT-B and one isolate displaying PT-A. All isolates contained SCC*mec* type II.

DISCUSSION

The data presented here provide information on frequency, species distribution and antimicrobial resistance of MRS in the nasal cavities of horses and humans and clinic environment samples in Turkey. The rate of nasal MRCNS carriage

Table 1. Occurrence and species distribution of MRS among horses, humans and environmental sites

MRS Species	Horses (n=209)	Veterinary staff (n=13)	Door handle (n=4)	Restraining box (n=2)	Medical equipment (n=8)	Total
S. lentus	96	12	4	1	6	118
S. sciuri	3	–	–	–	–	3
S. intermedius	1	–	–	–	–	1
S. fleuretti	1	–	–	–	–	1
Number of positive samples (%)	101 (48.3%)	12 (92.3%)	4 (100%)	1 (50%)	6 (75%)	123 (52.1%)

Table 2. Occurrence, species distribution, antimicrobial phenotype and genotype of MRS from horses, humans and clinic environment

Source	Staphylococcus species	Phenotype*	Genotype	Pulsotype	No. of isolates
Horse n=101	*S. lentus*	OXA, TE, E, STX, CIP, DA	*mec*A, *tet*M, *erm*A, *msr*A, *mph*C, *lnu*A	A2	1
	S. lentus	OXA, AMC, E, STX, CIP, DA	*mec*A, *erm*B, *erm*C, *mph*C, *lnu*A	A2	1
	S. lentus	OXA, TE, CN, MUP, DA	*mec*A, *tet*M, *aac(6')-aph (2")*, *lnu*A	A1	1
	S. lentus	OXA, CN, E	*mec*A, *aac(6')-aph (2")*, *mph*C	A1	1
	S. lentus	OXA, TE, E	*mec*A, *tet*K, *msr*A, *mph*C	B	1
	S. lentus	OXA, TE, DA	*mec*A, *tet*K, *tet*M, *lnu*A	A1	1
	S. lentus	OXA, AMC, TE, E	*mec*A, *tet*K, *erm*A, *mph*C	A1	1
	S. lentus	OXA, E, DA	*mec*A, *erm*A, *mph*C, *lnu*A	A1	1
	S. lentus	OXA, TE, E	*mec*A, *tet*K, *mph*C	A2	1
	S. lentus	OXA, TE, DA	*mec*A, *tet*K, *lnu*A	A1, A2	7
	S. lentus	OXA, TE	*mec*A, *tet*K, *tet*M	A1, A2	3
	S. lentus	OXA, TE, CN	*mec*A, *tet*K, *aac(6')-aph (2")*	A1, A2	3
	S. lentus	OXA, CN	*mec*A, *aac(6')-aph (2")*	A1	1
	S. lentus	OXA, DA	*mec*A, *lnu*A	A1, A2	6
	S. lentus	OXA, TE	*mec*A, *tet*K	A1, A2, B, C	44
	S. lentus	OXA, TE	*mec*A, *tet*M	B	1
	S. lentus	OXA	*mec*A	A1, A2, B	22
	S. sciuri	OXA, STX, FA	*mec*A, *fus*B	B	2
	S. sciuri	OXA, TE, DA	*mec*A, *tet*K, *lnu*A	A	1
	S. intermedius	OXA, TE	*mec*A, *tet*K	-	1
	S. fleuretti	OXA	*mec*A	-	1
Clinic staff n=12	*S. lentus*	OXA, TE, DA	*mec*A, *tet*K, *lnu*A	A1	6
	S. lentus	OXA, TE, DA, CN	*mec*A, *tet*K, *lnu*A, *aac(6')-aph(2")*	A1	2
	S. lentus	OXA	*mec*A	A1	4
Clinic environment n=10	*S. lentus*	OXA, TE, CN	*mec*A, *tet*M, *aac(6')-aph(2")*	A1	1
	S. lentus	OXA, TE, DA	*mec*A, *tet*K, *tet*M, *lnu*A	B	1
	S. lentus	OXA, TE, CIP	*mec*A, *tet*K	A1	1
	S. lentus	OXA, TE	*mec*A, *tet*K, *tet*M	A1	3
	S. lentus	OXA, TE	*mec*A, *tet*K	A1	3
	S. lentus	OXA	*mec*A	A1	1
Total					123

*OXA, oxacillin; DA, clindamycin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; AMC, amoxicillin-clavulanic acid; CN, gentamicin; MUP, mupirocin; FA, fusidic acid.

in horses (47.8%) was higher than those previously reported in the Netherlands (22%) [4], Japan (13 to 29.5%) [38, 39], Slovenia (42%) [34] and Italy (35.2%) [9] but lower than those reported in Denmark (50 to 82%) [2, 20]. In previous studies, colonization of horses with methicillin-resistant *S. lentus* and *S. sciuri* has been reported [2, 4, 9, 20, 38, 39]. However, to the best of our knowledge, this is the first report of *mec*A-mediated methicillin resistance in *S. intermedius* and *S. fleuretti*. Hesselbarth and Schwarz [11] suggested that horses might serve as a reservoir of *S. intermedius* for other animal species. Vernozy-Rozand *et al*. [35] were the first researchers to isolate *S. fleuretti* from cheese made from raw goat's milk in France. Since then, methicillin-resistant *S. fleuretti* has been isolated from the various sources including minced meat, bulk milk tanks, pig farmers, veterinarians, slaughterhouse employees, and animals (pigs and cows) [12].

In this study, a low frequency of resistance among MRS

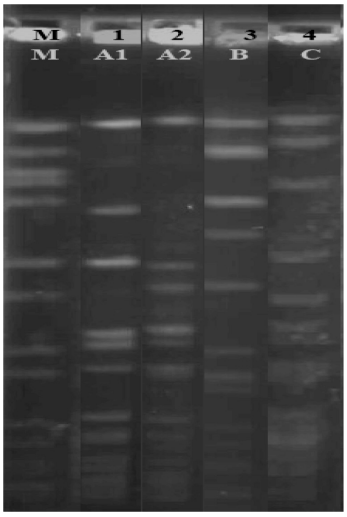

Fig. 1. PFGE analysis of *S. lentus* isolates. M: Molecular marker (*S. aureus* NCTC 8325). Lane 1, pulsotype A1 (63 strains); lane 2, pulsotype A2 (45 strains); lane 3, pulsotype B (5 strains); lane 4, pulsotype C (5 strains).

isolates towards clinically relevant antimicrobials such as ciprofloxacin, mupirocin, fusidic acid and amoxicillin-clavulanic acid was found. Only five isolates (4.1%) were found to be multidrug resistant. High tetracycline resistance (67.5%) was observed and was most likely due to routine use of this antibiotic in the hospital for antimicrobial therapy. Macrolide antibiotics are rarely used in equine medicine in Turkey but are used for the treatment of *Rhodococcus equi* infections in foals. Thus, it is not surprising to detect a low level of macrolide resistance, which was only observed in 7 isolates. Beta-lactam antibiotics and beta-lactam plus aminoglycoside combinations are among the most widely used antibiotics in equine clinics in Turkey. Weese *et al*. [36] reported that administration of these antibiotics during hospitalization has been considered a risk factor for MRSA colonization in horses. Schnellmann *et al*. [26] found that the use of penicillin suppresses natural and antibiotic susceptible microbiota, and poses a risk for colonization of multidrug-resistant microbiota on the skin of horses.

CNS pose a risk of horizontal transfer of the *mec*A gene to different staphylococci, especially *S. aureus*, leading the emergence of new MRSA strains. Therefore, it would be a significant public health issue if this agent was transmitted from animals to humans. For example, in Canada, an MRSA strain, classified as Canadian epidemic MRSA-5, has emerged in horses and was involved in a outbreak of skin infections in personnel in a veterinary hospital [36].

Recently, it has been reported that MRSA [3] and MRCNS [20] were acquired from equipment and the environment of equine hospitals. In the present study, MRCNS were isolated from hospital environments, including both human and equine contact surfaces. Environmental contamination of this agent is of importance due to the potential occupational risk for veterinary staff and students, and also for nosocomial equine infections [3].

The results of PFGE demonstrated that identical methicillin-resistant *S. lentus* and *S. sciuri* clones was shared by horses, humans and hospital environments, suggesting possible transmission between horses and humans directly or indirectly via contaminated hospital environments. These results are in accordance with the findings of Moodley and Guardabassi [20], who previously reported the presence of specific MRCNS clones among horses, humans and clinic environments at three equine facilities in Denmark.

The results of this study indicated that horses are a reservoir of MRCNS, which can potentially be transmitted between horses and humans. These strains are of increasing concern for veterinary and human medicine due to the emergence of MRSA strains by horizontal transmission of the *mec*A gene from MRCNS to *S. aureus*. Hence, continuous surveillance is important to monitor future emergence of new MRSA strains. In addition, effective infection prevention and control programs should be applied to reduce the amount of exposure of veterinary staff and the dissemination of MRS in veterinary hospital environments.

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