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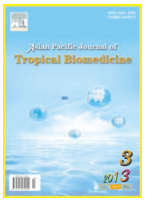

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A comparative evaluation of methicillin-resistant staphylococci isolated from harness racing-horses, breeding mares and riding-horses in Italy

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PEER REVIEW

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Comments

This is a good study in which the authors evaluated the distribution of staphylococcal species and levels of antibiotic resistance that were found to be different between isolates. Antibiotic pressure may lead to these differences. The results are interesting and suggested that MRS are present especially in harness racing-horses.
(Details on Page 172)

ABSTRACT

Objective: To investigate the prevalence of methicillin-resistant staphylococci (MRS) which is a potencial risk factor of transmission between animals and humans in different types of horses (harness racing-horses, breeding mares and riding-horses) and to compare the antimicrobial resistance of the isolates. **Methods:** A total of 191 healthy horses, housed at different locations of the Campania Region (Italy), were included in the study. Nasal swab samples were collected from each nostril of the horses. The *mecA* gene was detected by a nested PCR technique. Antibiotic susceptibility was tested for each isolate. **Results:** MRS was isolated from nasal samples of 68/191 (35.6%; 95% *CI*: 28.9%-42.9%) healthy horses. All isolates were coagulase-negative with the exception of two coagulase-positive MRS strains, identified as *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, 2/83 (2.4%; 95% *CI*: 0.4%-9.2%). Interestingly, both coagulase-positive MRS isolates were from harness racing-horses. These horses also presented a significantly higher positivity for MRS (53.3%; 95% *CI*: 40.1%-66.1%) than the breeding mares and riding-horses groups. Antibiotic susceptibility testing showed difference between isolates due to different origins except for an almost common high resistance to aminopenicillins, such as ampicillin and amoxicillin. **Conclusions:** It can be concluded that harness racing-horses may act as a significant reservoir of MRS as compared to breeding mares and riding-horses.

KEYWORDS

Methicillin-resistant staphylococci, Mares, Harness racing-horse, Riding-horse, Nasal swabs

1. Introduction

Methicillin resistance in *Staphylococcus aureus* (*S. aureus*) and coagulase-negative species is primarily mediated by the overproduction of PBP2a, an additional altered penicillin-binding protein with extremely low affinities for β-lactam antibiotics[1]. PBP2a is encoded by the gene *mecA*, residing on a large mobile genetic element designated staphylococcal chromosome cassette mec. Until now, seven major variants of staphylococcal chromosome cassette mec, type 1 to 7, have been described[2].

Methicillin-resistant *S. aureus* (MRSA) have been reported in almost all domesticated species, including dogs, cats, horses, cattle and sheep[3-6]. Skin and soft tissue MRSA infections[7], bacteraemia, septic arthritis and osteomyelitis[8,9], implant-related infections, metritis[10], omphalitis[11], catheter-related infections and pneumonia[12] have all been reported in horses.

In addition, *mecA*-harboring coagulase-negative staphylococci (CoNS) have been isolated from several domesticated and healthy animals[13,14]. In Japan, *mecA* positive CoNS were cultured from the skin and nares of healthy horses[15]. Recently, high prevalence of the CoNS was found in healthy horses in the Netherlands[16] and Slovenia[17] and in Italy[18].

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The increasing resistance of staphylococci to β-lactam antibiotics has become a major clinical problem and the strains also generally exhibited multiple resistance to tetracyclines, aminoglycosides, macrolides, lincosamides, and some other antimicrobial drugs[19-22]. Furthermore, since horses are often in close contact with their owners and farm staff members, the risk of transmission of these bacteria between animals and humans (or *vice versa*) must be taken into consideration. However, it is not well known the distribution of *mecA*-harboring staphylococci and the antimicrobial resistance with reference to the different habitats in which horses live. The aim of this study was to investigate the correlation between the prevalence of methicillin-resistant staphylococci (MRS), both coagulase-negative and coagulase-positive, from different standing of healthy horses in Italy, particularly in harness racing-horses, breeding mares and riding-horses.

2. Materials and methods

2.1. Sample collection

A total of 191 healthy horses, housed at different locations of the Campania Region (Italy) were sampled. The following are three different locations: (1) 60 harness racing-horses aged between 3-6 years randomly selected at the Agnano racetrack; (2) 64 breeding mares aged between 4-15 years from an Equine Reproduction Centre; (3) 67 horses aged more than 15 years from a large Riding Centre.

All samples were collected in the summer-autumn period of 2007 and these centres documented no history of MRSA. Owner's consent was obtained prior to enrolment of each horse. A cotton-tipped swab was collected from each nostril and kept at 4 °C (not longer than 24 h) in Amies medium until processing. Demographic information (age, breed, gender and use) was recorded for each horse at the time of sample collection.

2.2. Bacteriological assays

Nasal swab samples were plated on mannitol-salt agar and incubated aerobically at 37 °C for 24-48 h. Staphylococcal isolates were identified by colonial morphology, gram-stain, catalase and staphylo-coagulase (tube coagulase) reactions. Oxacillin (OX, methicillin) susceptibility test of all isolates was performed by OX disk diffusion method in accordance with Clinical and Laboratory Standards Institute-National Committee for Clinical Laboratory Standards (M31-A2, 2002). The OX susceptible *S. aureus* (ATCC 29213) and OX resistant *S. aureus* (ATCC 43300) strains were used as controls. Identification was confirmed with API-ID 32 Staph system (bioMérieux, Marcy L'Etoile, France) and methicillin-resistance was also confirmed by a positive PBP2a latex agglutination test (PBP2′ Test kit, Oxoid, Basingstoke, Hampshire, England). Stock cultures were stored at −70 °C in microbank vials (PRO-LAB Diagnostics, Richmond Hill, ON, Canada) for further analysis.

2.3. Extraction of DNA

Cells cultured in 1.5 mL of trypticase soy broth at 37 °C for 24 h were harvested and centrifuged at 16 000 r/min for 3 min. The pellet was washed with 1.0 mL of sterile distilled water, resuspended in 50 μL of Triton X-100 lysis buffer [100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA (pH 9.0), 10 g/L Triton X-100], boiled for 10 min, and then centrifuged at 16 000 r/min for 3 min. The suspension was cooled at room temperature for 5 min and centrifuged at 16 000 r/min for 3 min. The supernatant was used as template.

2.4. Detection of mecA gene by PCR

The *mecA* gene was detected by a nested PCR technique as described previously[15]. For the first amplification, the following primers were used (Invitrogen Ltd., Glasgow, UK): 5′-GTT GTT GTA GTT GTC GGG TTT GG-3′ (position 37 to 56, sense) and 5′-CCA CCC AAT TTG TCT GCC AGT TTC TCC-3′ (position 1 828 to 1 854, antisense); size of the amplified fragment was 1 818 bp. For the second (nested) amplification, the following internal primers were used: 5′-AGA TTG GGA TCA TAG CGT CA-3′ (position 375 to 394, sense) and 5′-GAA GGT ATC ATC TTG TAC CC-3′ (position 613 to 632, antisense); size of the amplified fragment was 258 bp. All PCRs were performed in duplicate. All PCR reagents were purchased from Invitrogen.

Briefly, PCR was performed in a 50 μL mixture containing template DNA, PCR buffer [10 mmol/L Tris-HCL (pH 8.4), 50 mmol/L KCl, 1.0 mmol/L $MgCl_2$], a 100 nmol/L concentration of each PCR primer, a 200 μmol/L concentration of each deoxyribonucleoside triphosphate, and 1.25 IU of *Taq* polymerase. Both reactions were performed in a DNA thermal cycler without mineral oil (Mastercycler Gradient Eppendorf, Eppendorf, Hamburg, Germany). The PCR and the nested PCR consisted of a preheating at 94 °C for 2 min, 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and incubation at 72 °C for 5 min. The final PCR products were loaded on a 1% agarose gel with 2 μg/mL of ethidium bromide to determine the size of the amplified products. As positive control, 100 ng of genomic DNA separated from a suspension of OX resistant *S. aureus* (ATCC 43300) was included in each experiment. Negative controls contained all reagents except DNA template.

2.5. Antibiotic susceptibility testing

Laboratory trials were performed in accordance with the principles described in the standard method of the National Committee for Clinical Laboratory Standards (2002), using the following antimicrobial agents: OX 10 μg, amoxicillin/clavulonic acid (AMC) 30 μg, ampicillin 10 μg, imipenem 10 μg, meropenem 10 μg, colistin sulfate 10 μg, cefaclor 30 μg, cefuroxime 30 μg, cefprozil 30 μg, ceftriaxone 30 μg, cefoxitin 30 μg, erythromycin 15 μg, lincomycin (MY) 2 μg, tetracycline 30 μg, doxycycline 30 μg, amikacin 30 μg, trimethoprim/sulphametoxazole 25 μg and ciprofloxacin 5 μg. The susceptibility of each isolate to the panel of antibiotics was revealed by the diameter size of the clear zones around the dish as directed by the manufacturer.

2.6. Statistical analysis

Statistical analysis was performed by the Student-Newman-Keuls Multiple Comparison test using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA).

3. Results

The staphylococcal isolates were tested for growth onto mannitol-salt agar; this assay was followed by other tests like gram-stain, catalase, staphylo-coagulase (tube coagulase) reactions and API-ID 32 Staph system. As for most cases, the CoNS grow and produce yellow colonies on mannitol-salt agar, it is necessary to perform further investigations in order to distinguish *S. aureus* from other staphylococci[23,24]. To evaluate the methicillin-resistance of all staphylococcal isolates, PBP2a latex agglutination test and PCR for *mecA* gene, a useful molecular marker of methicillin resistance, were performed.

From a total of 191 horses, 68 were carriers of bacterial strains phenotypically resisting to methicillin (35.6%; 95% *CI*: 28.9%-42.9%). Some horses were tested positive in only one nostril, precisely 53/191 (27.7%; 95% *CI*: 21.6%-34.8%), while 15/191 (7.8%; 95% *CI*: 4.6%-12.9%) horses presented different (8 horses) or similar (7 horses) MRS strains in both nostrils.

In Table 1, the identified species for each group are described. Species identified as *Staphylococcus lentus* (*S. lentus*) (24/30 isolates) and *Staphylococcus sciuri* (*S. sciuri*) (12/16 isolates) were mainly isolated from mares and riding-horses groups, respectively; while in the group of harness racing-horses different coagulase-negative MRS (CoN-MRS) species other than two strains of coagulase-positive MRS (CoP-MRS), precisely *S. aureus* and *Staphylococcus pseudintermedius* (*S. pseudintermedius*), were isolated [2/37 (5.41%); 95% *CI*: 0.9%-19.5%].

Table 1 also illustrates the positivity percentage of MRS in the three different groups of horses. The data of statistical analysis showed a significant difference ($P<0.05$) in the prevalence of MRS colonization between the three groups of examined horses. In any case, the harness racing-horses group presented a higher positivity (53.3%; 95% *CI*: 40.1%-66.1%) for MRS.

Table 2 illustrates the results of the antibiotic resistance of the strains isolated, *S. lentus* and *S. sciuri*. These isolates showed high resistance (100.0%) to β-lactams (OX, AMC, ampicillin); only the isolates from breeding mares group exhibited high resistance to OX and less resistance to AMC and ampicillin (10 μg). The sensibility to the carbapenem antibiotics meropenem, generally more potent against gram-negative bacteria, and imipenem, generally more potent against gram-positive bacteria[25], was high in *S. lentus* and *S. sciuri* isolated from the three different groups of horses. All isolates of *S. lentus* in riding-horses showed resistance to cephalosporin (cefaclor, cefuroxime, cefprozil, and ceftriaxone), macrolides (erythromycin and MY) and tetracycline (tetracycline and doxycycline), whereas *S. sciuri* were resistant only to MY as well as *S. lentus* and *S. sciuri* isolated in harness racing-horses.

Table 2
Percentage of resistance to 18 antimicrobial agents.

Antibiotics	Harness racing-horses		Breeding mares		Riding-horses	
	S. lentus	*S. sciuri*	*S. lentus*	*S. sciuri*	*S. lentus*	*S. sciuri*
OX	100.0	100.0	100.0	100.0	100.0	100.0
AMC	100.0	100.0	33.3	100.0	100.0	100.0
AMP	100.0	100.0	8.3	0.0	100.0	100.0
IMI	0.0	0.0	16.6	0.0	0.0	0.0
MRP	0.0	0.0	75.0	100.0	0.0	11.1
CEC	0.0	0.0	41.6	100.0	100.0	0.0
CXM	0.0	0.0	66.6	0.0	100.0	0.0
CPR	0.0	0.0	66.6	0.0	100.0	0.0
CRO	9.1	0.0	75.0	100.0	100.0	0.0
FOX	9.1	0.0	66.6	100.0	0.0	0.0
E	18.2	25.0	25.0	0.0	100.0	0.0
MY	100.0	100.0	58.3	100.0	100.0	100.0
TE	72.7	25.0	41.6	0.0	100.0	11.1
DXT	72.7	25.0	41.6	0.0	100.0	11.1
AK	18.2	8.3	0.0	0.0	0.0	0.0
SXT	0.0	0.0	0.0	0.0	0.0	0.0
CS	0.0	0.0	25.0	0.0	0.0	0.0
CIP	27.3	8.3	25.0	0.0	0.0	0.0

OX: oxacillin; AMC: amoxicillin/clavulonic acid; AMP: ampicillin; IMI: imipenem; MRP: meropenem; CEC: cefaclor; CXM: cefuroxime; CPR: cefprozil; CRO: ceftriaxone; FOX: cefoxitin; E: erythromycin; MY: lincomycin; TE: tetracycline; DXT: doxycycline; AK: amikacin; SXT: trimethoprim/sulphametoxazole; CS: colistin sulfate; CIP: ciprofloxacin.

4. Discussion

At present, MRSA, methicillin-resistant *Staphylococcus intermedius* and CoN-MRS infections represent one of the main problems in human and/or animal community[26,27]. It has been reported that many *Staphylococcus epidermidis* and other CoNS from human clinical isolates are resistant to methicillin[28]. An evolutionary theory proposed that *S. aureus* could have acquired *mecA* gene from *S. sciuri*, a species frequently isolated from animals, which harbours a close structural homologue of *mecA* gene[29]. These bacteria can serve as reservoirs of resistance determinants in the community, which could lead to the emergence of novel MRSA or MRS strains.

This study was designed to determine the prevalence of MRS, both coagulase-negative and coagulase-positive, isolated from nasal swabs of three different groups of healthy horses, precisely, harness racing-horses, breeding mares and riding-horses. A total of 68 *mecA* positive isolates were obtained, and 32, 24 and 12 positive horses for each group

Table 1
Detection of MRS isolated from nasal swabs of horses.

Horse type	Number of horses	Number of carriers	Number of isolates	Positive horses (%)	95% *CI* (%)	MRS
Harness racing horses	60	32	37	53.3	40.1-66.1	*S. sciuri* (12); *S. lentus* (11); *S. capitis* (5); *S. xylosus* (4); *S. aureus* (1); *S. auricularis* (1); *S. cohnii cohnii* (1); *S. hominis* (1); *S. pseudintermedius* (1)
Breeding mares	64	24	30	37.5	26.0-50.5	*S. lentus* (24); *S. xylosus* (4); *S. sciuri* (2)
Riding horses	67	12	16	17.9	10.1-29.6	*S. sciuri* (12); *S. xylosus* (3); *S. lentus* (1)

By the Student-Newman-Keuls Multiple Comparisons test, the prevalence of MRS was significantly different between the three groups of the examined horses. Number in the bracket indicates the number of isolates for each *Staphylococcus* species.

were observed respectively. Interestingly, harness racing-horses had a significant higher positivity ($P<0.05$) for MRS than breeding mares and riding horse groups. Yasuda *et al* demonstrated that the distribution of *mecA*-harbouring staphylococci may vary according to the circumstances of the horses[15], for example, breeding mares having less contact with humans and antibiotics than racing-horses and riding-horses. Furthermore, in Japan and Italy, riding-horses are commonly retired racing-horses. In our study, staphylococci which harboured the *mecA* gene responsible for methicillin resistance were isolated from 53.3% of racing horses, 37.5% of breeding mares and 17.9% riding-horses. The cause of a greater positivity for MRS in the first group of animals could surely be related with wider movements/activities and frequent human contact. Furthermore, our data about the greater number of MRS in breeding mares than in riding-horses are not in agreement with the report of Yasuda *et al.*[15], whose result is the opposite. This contrast is probably due to the matter that the breeding mares of our study are mares of an equine reproduction centre, and these mares were in movement and in contact with different horses and different farm staff members every month/year .

Also, species distribution of *mecA*-harboring staphylococci may vary according to the circumstances of the horses. The results of our study are in accordance with those of Yasuda *et al*[15]. Species identified as *S. lentus* and *S. sciuri* were more frequent in the mares and in the riding-horses groups, respectively, while in the harness racing-horses group, the number of staphylococcal species increases. In fact, *Staphylococcus xylosus, Staphylococcus capitis, Staphylococcus cohnii cohnii, Staphylococcus auricularis, Staphylococcus hominis*, and in addition two CoP-MRS, *S. aureus* and *Staphylococcus pseudintermedius,* were revealed.

After a horse was born, it may get normal flora, including *mecA*-harboring *S. sciuri*, from the mother mare and when it becomes a racing-horse, breeding mare or riding-horse, the flora of *mecA*-harboring staphylococci may change. In the three groups analyzed were also found differences in antibiotic resistance of strains isolated. Particularly, isolates from harness racing-horses and riding-horses had high resistances to several antibiotics, especially to lactams, while isolates from breeding mares had low or any resistance to β-lactams. This difference is probably due to the different amount of antibiotics used between these horse groups.

The evidence of animals as reservoirs of antimicrobial-resistant bacteria has already been reported[30]. Since horses are often in close contact with their owners and farm staff members, the risk of transmission of these bacteria between animals and humans (or *vice versa*) must be taken into consideration. Nasal carriage of MRSA in human is important in the epidemiology of human infections[31], whereas the importance of nasal carriage in animals is not clear. The role of nasal carriage of CoN-MRS in both humans and animals has not been elucidated yet.

In conclusion, unlike CoN-MRS, the prevalence of MRSA and methicillin-resistant *Staphylococcus intermedius* in healthy horses of the region Campania appears to be low. Furthermore, the greater sensitivity to MRS colonization in nasal passages, found in harness racing-horses group, could be due to movements and frequent human contact. Although the results reported here need further investigation, our data suggest that it is necessary to estimate the risk for the development of MRS especially in harness racing-horses.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Methicillin-resistant *S. aureus* (MRSA) has become a worldwide public health problem. The members of the *Staphylococcus* genus have a high frequency of conjugation and frequently acquire plasmids that encode antimicrobial resistance, and the production of the enzyme β-lactamase is the major mechanism by which staphylococci acquire resistance. The literature reports a higher interest for coagulase-positive staphylococci, especially for MRSA that has been reported in almost all domesticated animals. However, also the coagulase-negative staphylococci, which have long been considered as apathogenic, recently have assumed an important role as pathogens, and generally exhibite multiple resistance to antimicrobial drugs and high prevalence of the methicillin resistance.

Research frontiers

Studies are being performed in order to determine which are the significant reservoir of methicillin-resistant staphylococci between three different horse groups of the Campania Region (Italy). MRS, both coagulase-positive and coagulase-negative, have been isolated from nasal swabs of three groups of healthy horses, precisely, harness racing-horses, breeding mares and riding-horses.

Related reports

Our data about the greater number of methicillin-resistant staphylococci (MRS) in breeding mares than riding-horse are not in agreement with Yasuda *et al.* (2000), whose result is the opposite.This contrast is probably due to the matter that the breeding mares of our study are mares of an equine reproduction centre, then mares in movement, and in contact every month/year with different horses and different farm staff members.

Innovations and breakthroughs

Data regarding methicillin-resistant staphylococci (MRS) prevalence in different categories of horses are scarce. This study has showed that harness racing-horses had a significant higher positivity for MRS than breeding mares and riding-horse groups.

Applications

It may be significant to know the distribution of *mecA*-harboring staphylococci in horses. The results of the present study suggest that racing-horses may act as a significant reservoir of MRS as compared to breeding mares and riding-

horses. Thus, it is important to estimate and to monitor the presence of MRS especially in harness racing-horses.

Peer review

This is a good study in which the authors evaluated the distribution of staphylococcal species and levels of antibiotic resistance that were found to be different between isolates. Antibiotic pressure may lead to these differences. The results are interesting and suggested that MRS are present especially in harness racing-horses.

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