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Prevalence of Methicillin-resistant Staphylococci Isolates from Horses and Horse-related Personnel in Korea

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Abstract : Methicillin-resistant staphylococci (MRS) are emerging as important pathogens in humans and animals worldwide. The aim of this study was to investigate the prevalence of MRS in the racehorse population and in horse-related personnel in Korea. A total of 195 horses and 18 humans (eight veterinarians, three veterinary hospital staff, and seven horse-handlers) from racehorse farms in Korea were included in the study. The samples were collected from nasal cavities using bacterial transport medium and were cultivated on tryptic soy agar with 5% sheep blood for 3 days at 37°C to confirm the presence of *Staphylococcus* spp. Presumptive *Staphylococcus* spp. isolates were identified by 16S ribosomal RNA gene analysis. The coagulase test and oxacillin susceptibility tests were performed using the tube dilution and disk diffusion methods, respectively. The presence of the *mecA* gene was determined using a polymerase chain reaction assay. Of the 195 horses, 29 (15.6%) yielded 29 MRS isolates. Twelve (66.7%) of the 18 horse-related personnel yielded 12 MRS isolates. All of the MRS isolates from horses or horse-related personnel were identified as methicillin-resistant coagulase-negative staphylococci (MRCNS). The result of this study suggest that the prevalence of MRS increased with the duration of antibiotic use ($p = 0.002$). This study also provides evidence for the zoonotic transmission of MRCNS between horses and humans, although further investigations are needed.

Key words **:** racing horse, methicillin-resistant coagulase-negative staphylococci.

Introduction

Methicillin-resistant staphylococci (MRS) are important pathogens in humans and animals throughout the world (5). MRS are resistant to methicillin, which is mediated by the *mecA* gene. The gene encodes an extra PBP2a with a low affinity for β-lactams (6,9). Thus, β-lactam antibiotics are ineffective against staphylococci that express the *mecA* gene. Therefore, the *mecA* gene is considered to be a molecular marker of methicillin resistance in all staphylococci (11). It is assumed that *mecA* genes evolved in coagulase-negative staphylococci (CNS) before being distributed among other staphylococci (7). MRS are commonly resistant to multiple antibiotics and are a major clinical issue (4,9). MRS colonization may be a risk factor for antimicrobial resistance transmission between staphylococci and other bacteria (12). MRS transmission can occur between animals and humans, such as on horse farms where horses frequently make contact with veterinarians and horse-handlers. There have been numerous reports of methicillin-resistant coagulase-positive staphylococci (MRCPS) in humans and horses, especially methicillin-resistant *Staphylococcus aureus* (MRSA). However, little data is available from Korea. The existence of MRCNS was first reported in chickens in 1996, before MRCNS were isolated from horses in Japan in 2000 (14), in the Netherlands in 2006 (2), in Slovenia in 2006 (12), and in Italy in 2010 (6). The aim of the present study was to investigate the prevalence of MRS in the horse population and horse-related personnel in Korea.

Materials and Methods

Sample collection and culture

Nasal swab samples were taken from 195 healthy horses on a racing farm, including racehorses (153) and riding horses (42). A total of 18 humans who worked with horses, including eight veterinarians, three veterinary hospital staff, and seven horse-handlers, were also tested for the presence of MRS. All of the samples were collected during December 2012 and January 2013. One nasal swab was collected from each horse. A sterilized cotton-tipped swab was inserted approximately 10 cm into one nasal passage and withdrawn with the swab in contact with the nasal mucosa. A single swab was collected from each human. A sterilized cotton-tipped swab was inserted approximately to a depth of one nare passage while maintaining contact with the nasal mucosa. The swabs were placed immediately in Liquid Amies swab transport medium (Copan, Murrieta, CA, USA) and stored at 4°C

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until processing. Each sample was cultured in TSA II with 5% sheep blood (BD, Sparks, MD, USA) at 37°C under aerobic conditions. Pure bacterial colonies were isolated by repeated subculture. Yellow or white colonies were selected and tested for catalase. Catalase-positive colonies were gram-stained.

Coagulase test

The coagulase plasma tube test with rabbit coagulase plasma (BD, Sparks, MD, USA) was performed on the isolates that exhibited the typical staphylococcal colony morphology to distinguish coagulase-positive staphylococci from coagulase-negative staphylococci. Three to five colonies of each isolate were mixed with 0.5 mL of rabbit plasma and incubated at 37°C. Clot formation was checked at 4 h and 24 h.

Bacterial identification

Gram-positive staphylococcal isolates were confirmed by eubacterial 16S rRNA gene sequence. The DNA was extracted using a Qiagen DNA extraction kit, according to the manufacturer's instructions (Hilden, Germany). The 16S rRNA gene was amplified using primer pairs that corresponded to the eubacterial 16S rRNA genes, i.e., 5'-AACTGGAGGAAGGTGGGGAT-3' and 5'-AGGAGGTGATCCAACCGCA-3', as described previously by Mason *et al* (10). The polymerase chain reaction (PCR) amplification was performed in a total volume of 50 μL. The final reaction mixture contained the following: 50 mM KCl, 10 mM Tris-HCl (pH 8.3, 25°C), 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate (dNTP), 100 ng of each primer, and 5 units of Taq polymerase (iNtRON Biotechnology, Sungnam, Gyeonggi, Korea). PCR was performed by using a TaKaRa Thermal Cycler Dice (Takara Bio Inc., Otsu, Shiga, Japan) under the following conditions: an initial denaturation at 94°C for 3 min, followed by 36 cycles at 94°C for 1.5 min, 53°C for 30 s, and 55°C for 1 min, with a final incubation at 72°C for 10 min. The PCR products were separated by electrophoresis for 50 min at 100 V on a 2% agarose gel and were stained with ethidium bromide to facilitate visualization under ultraviolet light. The amplicons were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (PE Applied Biosystems, Foster City, CA, USA). Staphylococci were identified based on comparisons with the 16S rRNA gene sequences deposited in GenBank.

Methicillin resistance tests

All of the isolated colonies were subjected to methicillin susceptibility testing using the oxacillin disc diffusion method (ODD), where a 1 μg oxacillin disc (BD, Cockeysville, MD, USA) was placed on Mueller-Hinton agar (BD, Sparks, MD, US). The zone of inhibition was checked after incubation for 24 h at 35-37°C under aerobic conditions. The methicillin resistance, determined according to the ODD method, was defined according to the method of the Clinical and Laboratory Standards Institute (M13-S17, 2007). The *mecA* gene was also subjected to PCR to determine the presence of methicillin resistance genes. The *mecA* gene was amplified using M1 and M2 primers. The PCR conditions were the same as those described previously, i.e., the primers were *mecA1* (5'-TCCAGGAATGCAGAAAGACCAAAGC-3'), and *mecA*2 (5'-GACACGATAGCCATCTTCATGTTGG-3'). Any positive results detected by the ODD method and *mecA* gene PCR were regarded as methicillin-resistant isolates.

Statistical analysis

The means and standard deviations of all values were calculated using Microsoft Excel. The relationship between the presence of MRS and groups was evaluated using Fisher's exact test with SAS (Statistical Analysis System). The relationship between the presence of MRS and the duration of antibiotic usage, which was evaluated using Wilcoxon's Signed-Rank test with SAS. $p < 0.05$ was considered significant.

Results

The prevalence of staphylococci is shown in Table 1. Staphylococci were isolated from 64 (32.8%) of 195 horses and 14 (77.8%) of 18 horse-related personnel based on the results of the 16S rRNA gene PCR assay and sequence analysis. All of the staphylococcal isolates were coagulase-negative; thus, they were considered MRCNS in all of the horses and horse-related personnel.

Staphylococci that exhibited methicillin-resistant positive reactions according to the ODD method or *mecA* gene PCR were regarded as MRS in this study. Sixteen (39.0%) of 41

Table 1. The prevalence of staphylococci in horses and horse-related personnel

Types		No. of samples	No. of staphylococcal samples	Prevalence (%)
Horses (n = 195)	Racehorses	153	48	31.4
	Riding horses	42	16	38.1
	Total	195	64	32.8
Horse-related personnel (n = 18)	Veterinarians	8	8	100.0
	Horse-handlers	7	5	85.7
	Hospital staff	3	1	33.3
	Total	18	14	77.8

Table 2. Results of the oxacillin disc diffusion (ODD) test and *mecA* gene PCR performed on methicillin-resistant staphylococci (MRS) isolates from horses and horse-related personnel

Tests	Number of MRS isolates	Positive rate
ODD (+) *mecA* (+)	16/41	39.0%
ODD (+) *mecA* (–)	13/41	31.7%
ODD (-) *mecA* (+)	12/41	29.3%

Table 3. Detection of methicillin-resistant staphylococci (MRS) isolates from horses

Type	Number of horses	MRS		
		CPS*	CNS**	Positive rate
Racehorses	48	0	24	50.0%
Riding horses	16	0	5	31.3%
Total	64	0	29	45.3%

*CPS = coagulase-positive staphylococci.
**CNS = coagulase-negative staphylococci.

Table 4. Methicillin-resistant staphylococci (MRS) positive rates according to the jobs of the horse-related personnel

Job	Number of human subjects	MRS		
		CPS*	CNS**	Positive rate
Veterinarian	8	0	7	87.5%
Horse-handler	7	0	4	57.1%
Hospital staff	3	0	1	33.3%
Total	18	0	12	66.7%

*CPS = coagulase-positive staphylococci.
**CNS = coagulase-negative staphylococci.

Table 5. Relationships between the presence of methicillin-resistant staphylococci (MRS), the duration of antibiotic usage, and the ages of horses

Methicillin resistance	No. of horses	Days of antibiotic usage*	Age
Non-MRS horses	35	3.2 ± 4.6^{a}	5.7 ± 3.8
MRS horses	29	6.8 ± 8.5^{b}	5.3 ± 3.6

*Number of days when antibiotics were used in the past 3 years (penicillin, ceftiofur, and gentamicin). $^{a,b}p = 0.002$.

MRS samples were positive using both methicillin resistance methods. Thirteen (31.7%) of 41 MRS samples were positive in the ODD test and 12 (29.3%) of 41 MRS samples were positive according to the *mecA* gene PCR (Table 2).

Twenty-nine out of 64 horses with staphylococci were positive for MRS (Table 3). The MRS-positive rates were not statistically different between the two horse groups, i.e., 24 out of 48 (50.0%) in racehorses and five out of 16 (31.3%) in riding horses.

MRS prevalence in horse-related personnel differed according to job type, i.e., 7/8 (87.5%) were veterinarians, 4/7 (57.1%) were horse-handlers, and 1/3 (33.3%) were veterinary hospital staff (Table 4).

The duration of antibiotic usage and the ages of horses were also investigated. The number of days when antibiotics were used during the previous 3 years was 6.8 ± 8.5 days in the MRCNS horses group and 3.2 ± 4.6 days in the non-MRCNS horses group. The prevalence of MRS increased with the duration of antibiotic use ($p = 0.002$). However, the horse age was no significantly difference between the two groups (Table 5).

Discussion

This study showed that MRS are present in healthy horses and horse-related personnel in Korea. All of the MRS isolates were coagulase-negative and no MRCPS were isolated, including MRSA, which is a major clinical problem for human and veterinary medicine. In the present study, the MRCNS rate (66.7%) in horse-related personnel was higher than that in samples from horses (45.3%). These data agree with those of previous report, where the rates of MRCNS were 22.5% in horses and 35.7% in humans (2). The high MRCNS prevalence in humans in the present study may be related to frequent contacts with different horses. The samples were collected from a racing farm where many horses moved in and out on a frequent basis and a limited number of authorized horse-related personnel handled the horses. The horse-related personnel came into contact with many horses on a frequent basis because of conditions on the racing farm. Frequent contact with horses is a potential risk factor for MRS transmission (6). Interestingly, MRCNS prevalence differed depending on the jobs of the horse-related personnel. The MRCNS-positive rates were highest among veterinarians who came into contact with horses most frequently, followed by horse-handlers who had contact with a limited number of horses in the stables, and veterinary hospital staff who had the least contact with horses (Table 4). These results suggest that MRCNS can be transmitted from horses to humans.

The MRCNS-positive rate in racehorses was slightly higher than that in riding horses. This may have been due to stress and several other factors such as environmental differences, training activities, and frequent antibiotic administration. The administration of antibiotics appears to be a risk factor for MRSA colonization in animals (13). To the best of the author's knowledge, this has not been elucidated in the case of MRCNS. The number of days when antibiotics were administered to the horses in the previous three years was investigated. The most commonly used antibiotics in Korean clinics were penicillin, ceftiofur, and gentamicin. MRS usually produce aminoglycoside-modifying enzymes, which makes them aminoglycoside resistant (3). The present study was performed over a longer period than that of a previous study that also investigated antibiotic administration in horses (13). MRCNS-positive horses had a higher mean number of days of antibiotic administration than that of MRCNS-negative

horses. These data are consistent with the results reported by Weese *et al* (13) who investigated antimicrobial administration in the previous 30 days as a possible risk factor for MRSA. Like MRSA, the administration of antibiotics also appeared to be a risk factor for MRCNS. Overall, these results suggest that humans who came into contact with MRCNS-colonized horses had an increased risk of MRCNS.

Methicillin resistance was tested using two methods, i.e., the ODD test and *mecA* gene PCR. Both tests can detect methicillin resistance, but the results of the two methods were not identical in this study. This was possibly due to a combination of several factors, such as the different specificities of methicillin resistance tests, heterogeneous resistance, and borderline resistance. The specificity of ODD is relatively low (average 80%), whereas the *mecA* gene PCR assay is considered to be the gold standard method (1,3). Methicillin resistance is heterogeneous in staphylococci, although most individual cells are susceptible to methicillin and only a small portion of cells are methicillin resistant, which means that most isolates will exhibit some degree of methicillin resistance (3). Borderline resistance is characterized by the overproduction of staphylococcal β-lactamase or modifications of the normal penicillin-binding protein in *mecA* gene-negative staphylococci and it is extremely heterogeneous in *mecA* gene-positive staphylococci (3). Therefore, variable methicillin resistance results were obtained, regardless of the presence of the *mecA* gene in the present study. It may be clinically meaningful to differentiate methicillin-resistant isolates using both tests.

MRSA, a coagulase-positive staphylococcus, has frequently been reported as an important pathogen in human and veterinary medicine (8,13). MRSA emerged when methicillin-susceptible *Staphylococcus aureus* acquired the *mecA* gene from CNS (8). Presumably, MRSA is present in the horse population in Korea, although coagulase-positive staphylococci were not isolated in this study. MRCNS can serve as reservoirs of methicillin resistance, which can lead to the emergence of MRSA or MRCPS, and the presence of MRSA in human medicine has been reported in Korea (4,6). Therefore, the prevalence of MRSA in horses in Korea should be addressed in further investigations.

In conclusion, MRCNS are adapted to the horse and human bacterial flora in Korea, and it seems that zoonotic transmission has occurred. The colonization of horses by MRCNS may be an additional concern because horses may transmit infectious pathogens to humans. Further research is required to determine the relationships between MRCNS colonization and MRCNS infections of horses and humans.

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말 및 말관련 종사자의 methicillin 내성 포도상구균의 유병율 조사

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요 약 : Methicillin 내성 포도상구균은 전세계적으로 사람과 동물에서 중요한 병인체로 주목 받고 있다. 본 연구는 국내 말과 말을 취급하는 사람에서의 methicillin 내성 포도상구균 발생현황을 조사하고자 실시하였다. 국내 경주마 목장에 소재하는 총 195두의 말과 18명의 말을 취급하는 사람(8명의 수의사, 7명의 말 관리사, 3명의 동물병원 직원)을 대상으로 하였다. 면봉을 이용하여 한쪽 비강에서 시료를 채취하여 세균수송배지에 보관 후 5% 양 혈액배지에서 37°C 3일간 배양하여 포도상구균 존재여부를 확인하였다. 포도상구균은 16S rRNA 유전자 분석을 실시하여 동정하였으며, 동정된 포도상구균은 coagulase 검사를 실시하였다. Methicillin 저항성을 확인하기 위하여 oxacillin 디스크 검사와 함께 *mecA* 유전자 존재를 PCR을 통하여 확인하였다. 검사를 실시하였던 말 195두 중 64두가 포도상구균으로 동정되었으며, 이중 29두(44.6%)가 methicillin 내성 포도상구균으로 확인되었다. 말을 취급하는 18명 중 14명의 시료에서 포도상구균이 동정되었으며, 이중 12명(85.7%)의 시료에서 methicillin에 내성을 가지고 있는 포도상구균으로 확인되었다. 말과 사람에서 동정된 모든 methicillin 내성 포도상구균은 coagulase 음성으로 확인되었다. 또한 항생제의 사용기간이 긴 개체에서 사용기간이 짧았던 개체군보다 methicillin 내성 포도상구균이 높은 것으로 나타났다($p = 0.002$). 본 연구 결과는 사람과 말 사이에서 인수공통전파가 일어날 수 있음을 시사한다.

주요어 : 경주마, methicillin 저항성 포도상구균