ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS* SPP. ISOLATED FROM RIDING-HORSES NASAL MUCOSA

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Abstract. The aim of this study was to analyse *Staphylococcus* species distribution in the upper respiratory tract of riding horses and to determine antimicrobial resistance of the isolated staphylococci. Two hundred and ten isolates of *Staphylococcus* spp. were obtained (78.7 %) from the 267 healthy horses tested. A wide variety of the species was detected while the most prevalent were *S. aureus* (42.9 %), *S. lentus* (14.3 %), *S. pseudintermedius* (13.1 %), *S. cohnii* (9.5 %) and *S. xylosus* (9.5 %). Other species such as *S. sciuri, S. warneri* or *S. vitulinus* were detected only in a few cases. Eighty four isolates (40 %) demonstrated resistance to at least one antimicrobial. The most frequent resistance was demonstrated to penicillin G (66.7 %) and erythromycin (41.6 %). No strains resistant to vanocmycin, daptomycin and co-trimoxazole were detected. Ten isolates demonstrated phenotypical resistance to methicillin although only six of them (two isolates of *S. lentus* and the same amount of *S. sciuri* and *S. vitulinus*) carried the *mec*A gene while the *mec*C gene was not detected. In spite that resistance to macrolides and aminoglycosides was prevalent (35 and 5 isolates respectively), no genes encoding resistance to those classes of antibiotics (*aac(6')-Ie-aph(2')-Ia, aph(3')-III*, *aph(2')-Ic)* and macrolides (*msr*AB, *erm*A and *erm*C) were detected. Resistance to tetracycline was demonstrated in 33.2 % of the isolates attributed to *tet*K and *tet*M genes. This study revealed the first occasion of methicillin-resistant *Staphylococcus* spp. among horses in Lithuania.

Keywords: *Staphylococcus* spp., horse, antimicrobial resistance genes, multidrug resistance

Introduction. Staphylococci are one of the major groups of bacterial commensals isolated from skin, skin glands, and mucous membranes of mammals. S*taphylococcus aureus* is a member of the commensal microbiota and a common opportunistic pathogen in many animal species, including humans and horses. Colonization, wherein *S. aureus* resides at a body site without producing clinical disease, is more common than clinical infection in humans and other species. While *S. aureus* has traditionally been an important pathogen, its role in disease has increased since the emergence of methicillin-resistant strains (Burton et al., 2008; Bergström et al., 2012). As E. Van Duijkeren et al. (2004), Van den Eede et al. (2009), Karakulska et al., (2012) and other researchers maintain that the emergence and dissemination of antimicrobial resistance among staphylococci is an important problem in human and veterinary medicine. Some of researchers are sure that in particular methicillin-resistant *S. aureus* (MRSA) is a tremendous concern in human medicine worldwide (Cuny et al, 2006; Bergström, 2012) and is an emerging problem in veterinary species. The resistance of methicillin in staphylococci is mediated by the *mecA* gene – a gene that encodes a novel penicillin binding protein (PBP2a). MRSA are resistant to all beta-lactam antimicrobials. This is caused by the production of an altered penicillin-binding protein encoded by the *mec*A gene (Weese et al., 2005, Baptiste et al., 2005; Burton et al., 2008). The MRSA strains are often resistant to many other antimicrobials as well, and that limits treatment options. O'Mahony et al. (2005), Busscher et al. (2006), Leonard (2008) reports, that MRSA has been identified as a cause of disease in a variety of animal species including horses and dogs in Canada, the United States, the United Kingdom, the Netherlands and Ireland, and transmission of MRSA between humans and animals was identified. MRSA potential for zoonotic transmission is lately on active investigations, because colonization by methicillin-resistant staphylococci of any species may pose a risk for plasmid-encoded transfer of antimicrobial resistance determinants between staphylococci and other bacterial organisms (Yasuda et al., 2000; Bellacicco et al., 2009). Emergence of MRSA as an equine pathogen is of additional concern because horses may be a community reservoir of MRSA and a source of infection or reinfection for persons. Also, the presence of methicillin-resistant staphylococci in animals in the community may pose a danger to veterinary care facilities. There can be projected the potential for adaptation of endemic methicillin-resistant staphylococci to animal species. However, Burton et al., (2008) reported, that MRSA colonization was not identified in any of 497 horses from Atlantic Canada. Methicillin-susceptible *S. aureus* (MSSA) was isolated from a subsample of 7.9 % horses. Colonization with MSSA is relatively common in healthy horses in Atlantic Canada, but MRSA is currently rare or absent (Weese et al., 2005). Also Tom Maddox et al. (2010) reported, that coagulase-negative *Staphylococcus* spp. (CNS) is more common in horses (*S. scuiri*). The epidemiology of staphylococcal diseases in domestic animals is not well understood. Little is known, however, about their origin and the general equine MRSA colonization status. In West Europe, and Lithuania in particular, neither the colonization rate nor the present strains or their antimicrobial susceptibility patterns in horses are known.

The aim of this study was to analyse the staphylococcal flora of the nasal cavity of healthy horses and determine phenotypic and genotypic resistance to antimicrobial agents.

Material and methods. *Sample collection*. The research was performed in 16 Lithuanian horse stables, from April to June in 2013. Stables were situated in Kaunas, Vilnius, Klaipeda and Šiauliai districts. All stables were used for keeping riding horses and for public/sport riding purposes as well. The stables were picked randomly to represent the general horse population. Approximately 15 horses were sampled from each stable. A total 267 healthy riding-horses aged between 5–15 years were sampled. A single nasal swab was collected from each horse. For each horse, a cotton-tipped culture swab was inserted approximately10 cm into nasal passage and retracted in contact with nasal mucosa. Samples were collected using sterile Amies media swabs (MWE, UK) under aseptic conditions. The samples at 4°C were delivered to the laboratory within 6 hours for processing.

Isolation and identification of staphylococci. The clinical material was inoculated onto Columbia Blood Agar containing 5 % sheep blood (Oxoid, UK), Mannitol Salt Agar (Liofilchem, Italy), Contrast MRSA Broth (Oxoid, UK), and Brilliance MRSA 2 Agar (Oxoid, UK). Staphylococci up to the genus level were identified according to morphology charactersitics, catalase production, gram-staining, susceptibility to furazolidone. (Glenn Songer, Post Karen W., 2004; Qiunn et al, 1993). Species identification was performed according to pigment and coagulase production, presence of protein A and clumping factor (Microgen StaphPLIUS, UK), and biochemical properties by using identification system RapID Staph Plus (Remel, USA).

DNA extraction. DNA material for molecular testing was obtained after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance (CRL-AR, 2009) with slight modifications. Briefly, bacterial colonies were taken with a bacteriological loop from the surface of Mueller Hinton Agar (Oxoid, UK) and transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min. Then the supernatant was discarded and the pellet was re-suspended in Tris-EDTA (TE) buffer (Fluka, Switzerland). The suspension was heated using thermomixer (Biosan, Latvia) to 100 °C for 10 minutes. The boiled suspension was transferred directly on ice and diluted by 1:10 in TE.

Molecular methods for taxonomic verification. Genus specific 16S as well as species-specific thermonuclease (*nuc*) genes were investigated by PCR with positive control. (Poulsen A. B. et al., 2003).

For complicated cases, 16S rRNA sequencing for species confirmation was performed using ABI3730XL sequencer. Bacterial DNA was prepared by DNA Clean Concentrator 25 kit (Zymo Research, USA) following manufacturer recommendation. Sequences were analyzed using Molecular Evolutionary Genetic Analysis software (MEGA, version 6). Basic local alignment search tool (BLAST) was used for comparison of obtained sequences with sequences presented in the database of National Center of Biotechnology Information (NCBI, 2014).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method (Quinn et al., 1993). Antimicrobial discs were provided by Liofilchem (Italy). Resistance to penicillin (1 IU), cefoxitin (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), tetracycline (30µg), erythromycin (15 µg) and trimethoprim/sulfamethoxazole (25 µg) was tested by incubation (37º C, 24 h) with Mueller-Hinton (Oxoid, UK) agar. Inoculum standardization, medium and incubation conditions as well as interpretation of inhibition zones were performed according to the manufacturer's guidelines (Liofilchem, Italy). Antimicrobial quantitative susceptibility (MIC) testing was performed using the broth microdilution method. Sensititre® plates (Thermo Scientific, UK) and ARIS 2X (Thermo Scientific, UK) automated system were used with the following antimicrobials: ceftriaxone, daptomycin, ciprofloxacin, clindamycin, erythromycin, gentamicin, levofloxacin, linezolid, oxacillin, penicillin, quinupristin/dalfopristin, tetracycline, trimethoprim/ sulphamethoxazole and rifampin. Sensititre® antimicrobials plates were inoculated according to manufacturer standart recommendations. Interpretation of results was carried-out using manufacturers software SWIN® (Thermo Scientific, UK) adapted to clinical breakpoints of Euopean Committee on antimicrobial susceptibility testing (EUCAST). The quality control strain *Staphylococcus aureus* ATCC 29213 was included in each assay for validation purposes.

PCR assay for antimicrobial genes. Detection of genes encoding to antimicrobial resistance *(mec*A, *bla*Z, *tet*K, *tet*M, *erm*A, *erm*C, *msr*A/B, *aac(6')-Ie-aph(2")-Ia, aph(3')-IIIa*) was performed. Annealing temperatures and oligonucleotides used are presented in Table 1.

Statistical analysis. Statistical analysis was performed using "R 1.8.1" package (http://www.r-project.org/). The categorical variables were comparedas by the chi-square test and Fisher's exact test. The results were considered statistically significant if $p<0.05$.

Results. Two hundred and ten samples from 267 tested (78.6 %) horses were positive for the presence of *Staphylococcus* spp. In all cases, only one *Staphylococcus* spp. isolate was obtained from each horse. According to the quality research data, 84 staphylococci strains (40.0%) showed resistance to at least one antimicrobial. Fig. 1 presents Staphylococcus species distribution among these staphylococci isolates.

As it is shown in Fig. 1, *S. aureus* was the most common ($P<0.05$) isolate (36). *S. lentus* (12) and *S. pseudointermedius* (11) isolates were identified respectively. Only two *S. sciuri,* and two *S. warneri isolates* were identified ($P<0.05$).

MIC research data of selected *Staphylococcus* spp., are presented in Fig. 2. The resistance patterns show *Staphylococcus* spp. strains to be highly resistant to penicillin G ($P<0.05$), erythromycin or tetracycline.

Penicillin resistant staphylococci often showed resistance to other antibiotics as well. Among 84 representative isolates, 26.2 % ($P<0.05$) showed resistance to penicillin only and 66.7 % showed multiple resistances to antimicrobials including penicillin, erythromycin, tetracycline, ciprofloxacin, and gentamicin (Fig. 2). The least number ($P<0.05$) of all *Staphylococcus* spp. isolates were resistant to ciprofloxacin and gentamicin (4.8 %).

It is taken that multi-resistant strains are resistant to 3 antibiotic class drugs and more (in the present work mainly multi-resistant strains are taken into consideration). Multi-resistant staphylococci were present in 13.1 % of 84 selected isolates. All *Staphylococcus* spp. isolates were sensitive to sulfametoxazol/trimetoprim.

Table 1. **Oligonucleotide primers used in this study**

Primer name	Sequence (5' - 3')	Size, bp and T(ºC)	Target gene	Source
mecA1	GGGATCATAGCGTCATTATTC	527 (61)	*mec*A	CRL-AR, 2009
mecA2	AACGATTGTGACACGATAGCC			
mecC1	AAGTTAATCAAAAATGGGTTCAGC	304 (50)	*mec*C	Harrison et al., 2012.
mecC2	GGTTGTAATGCTGTACCAGATCC			
blaZ1	CAGTTCACATGCCAAAGAG	772 (50)	*bla*Z	Schnellmann et al., 2006
blaZ2	TACACTCTTGGCGGTTTC			
tetM1	GTTAAATAGTGTTCTTGGAG	656 (45)	*tet*M	Aerestrup et al., 2000
tetM2	CTAAGATATGGCTCTAACAA			
tetK1	TTAGGTGAAGGGTTAGGTCC	718 (55)	*tet*K	Aerestrup et al., 2000
tetK2	GCAAACTCATTCCAGAAGCA			
aac6-aph2F aac6-aph2R	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	348 (61)	*aac(6')-Ie-aph(2'')-Ia*	Perreten et al., 2005
aph3-IIF	CCGCTGCGTAAAAGATAC	609 (57)	*aph(3')-IIIa*	Perreten et al., 2005
aph3-IIR	GTCATACCACTTGTCCGC			
ermA1	AAGCGGTAAAACCCCTCTGAG	442 (53)	*erm*A	Jensen et al., 2002
ermA2	TCAAAGCCTGTCGGAATTGG			
ermC1	ATCTTTGAAATCGGCTCAGG	295 (48)	*erm*C	Jensen et al., 2002
ermC2	CAAACCCGTATTCCACGATT			
msrAB1	GCAAATGGTGTAGGTAAGACAACT	350 (55)	*msr*A/B	Thumu et al., 2012
msrAB2	ATCATGTGATGTAAACAAAAT			

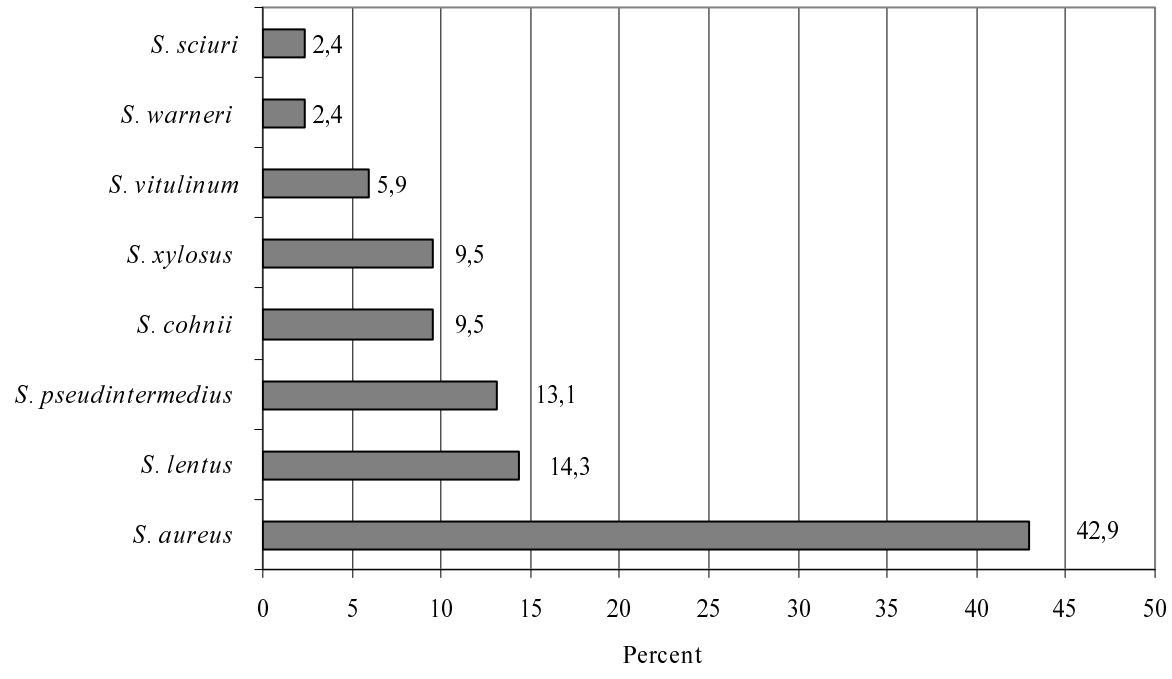

Fig 1. ***Staphylococcus* species (%) resistant to at least one antimicrobial isolated in horses** (n=84)

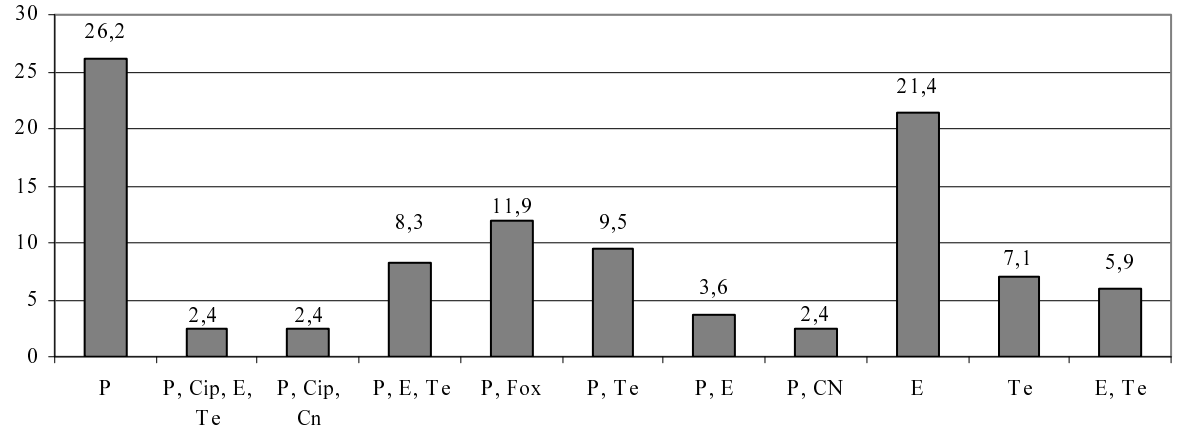

Fig 2. **Antibiotic resistance patterns** (%) **of *Staphylococcus* spp. isolates** (n=84)
CN – gentamicin, TE – tetracycline, Cip – ciprofloxacin, P – penicillin, E – erythromycin, Fox – cefoxitin

Ten *Staphylococcus* spp. strains were resistant to penicillin and cefoxitin. They showed phenotypical resistance to beta lactams class antimicrobials only. Genes encoding resistance to separate classes of antimicrobials were found in different numbers (Table 2). The most prevalent genes were *mec*A, *bla*Z and *tet*K. *tet*M resistance genes were few. Further study (PCR) (Table 2) showed presence of *mec*A gene in six isolates: two *S. lentus*, two *S. sciuri* and two *S. vitulinum*. Macrolide resistance encoding genes *aac*, *aph*, *msr*A/B, *erm*A, *erm*C were not detected. Three CNS strains had genes (*tet*K, *tet*M) encoding resistance to tetracycline. Strains with more than one resistance gene were not detected.

Six isolates with detected *mec*A gene were resistant to tetracycline, gentamicin and ciprofloxacin. MIC distribution of these isolates to penicillin was 0.25–4 mg/l, oxacillin – 4–8 mg/l (Table 3). Half of these strains were also resistant to clindamycin, but not to erythromycin, MIC to tetracycline were 2 mg/l.

Table 2. **Resistance genes identified in the *Staphylococcus* isolates** (n=84)

Target gene	S. aureus	CNS
*mec*A	0	6
*mec*C	0	0
*bla*Z	2	0
*tet*K	0	2
*tet*M	0	1
aac(6')-Ie-aph(2'')-Ia	0	0
aph(3')-IIIa	0	0
*erm*A	0	0
*erm*C	0	0
*msr*A	0	0

Table 3. **MIC distribution of *Staphylococcus* spp. strains** (n=6)**, with detected *mec*A gene**

Antimicrobial drug	MIC Distribution, mg/L													
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Ciprofloxacin						4	2							
Clindamycin		1	2	3										
Daptomycin			4		1	1								
Erythromycin			4											
Gatifloxacin			3		3									
Gentamicin						6								
Levofloxacin				4	2									
Linezolid					2	4								
Oxacillin+2% NaCL							2							
Penicillin			2	2		1	1							
Quinupristin/dalfopristin				3	3									
Rifampin		2		4										
Tetracycline						6								
Trimethoprim/sulphamethoxazole					6									
Vancomycin					6									
P. S. Green – sensitive, yellow – intermediate, red - resistance														

A *bla*Z resistance gene were detected in two *S. aureus* strains. These strains showed resistance to penicillin – 0.06mg/l, oxacillin – 0.5mg/l, but *mec*A gene were not detected. CNS (*S. xylosus*) strains, which contain *tet*K and *tet*M genes, showed resistance (MIC) to oxacilline at 0.25mg/l, gentamicin – 2mg/l and erythromycin – 4mg/l.

Discussion. Staphylococci represent a normal flora in both humans and animals. It often colonizes the skin and nose in healthy individuals; however, it can also cause severe diseases. Many researchers have reported that MRSA and methicillin-resistant coagulase negative staphylococci (MRCNS) are an important cause of infections in humans and animals (Deurenberg and Stobbering, 2008; Haeni et al., 2010). The most important strain among the *S. aureus* is the methicillin resistant *S. aureus*. MRSA are multidrug resistant *S. aureus* and it is the major pathogen causing nosocomial infections. Reports about significant clusters of MRSA in horses have come from Canada (Weese et al., 2005), USA (Hartmann et al., 1997), Ireland (O'Mahony et al., 2005), Austria (Cuny et al., 2006) and Malaysia (Zunita et al., 2008). Baptiste et al. (2005) indicated, that methicillin-resistant *S. aureus* is as MRSA seems to be emerging as an important equine pathogen. Busscher et al. (2006), Vengust et al. (2006) in their studies have reported colonization rates of 0–16 % in horses, 13 % in people that work with horses, and 9.7 % in veterinary hospital personnel working in large animal clinics. Meanwhile in our research, based on analysis of a big number of horses kept in Lithuania, only less than a half (42.6 %) of identified *Staphylococcus* spp. were *S. aureus. S. pseudintermedius* was identified in 13.1 %, i.e. coagulase positive *Staphylococcus* spp. (CPS) were identified in 55.7 % of cases what is a little bit more than CNS (44.3 %). Our research showed a quite frequent occurrence of *S. lentus*, *S. cohnii*, *S. xylosus*, *S. vitulinum*, *S. sciuri*, and *S. warneri.* The researchers from Japan also reported the data which are very close to the data of our research. The isolates were identified as 6 species (*S. epidermidis, S. lentus, S. saprophyticus, S. xylosus, S. sciuri,* and *S. haemolyticus*). They report, that MRSA was seldom isolated (Yasuda et al., 2000). They also identified, that 29.5 % of yielded isolates were methicillin-resistant staphylococci. Corrente et al. (2009) also reported that methicillin-resistant *Staphylococcus epidermidis* strain was isolated from a saddle horse affected by osteolysis. MRCNS were isolated from 78.8 % of horses housed in the same riding club.

We found fewer MRCNS in our research. Moreover, according to the data of other researchers, such as Burton et al. (2008), MRSA colonization was not identified in any of 497 horses from Atlantic Canada. MSSA was isolated from a subsample of 7.9 % horses. Under the opinion of the researcher, colonization with MSSA is relatively common in healthy horses in Atlantic Canada, but MRSA is currently rare or absent.

In our research, for the first time the presence of methicillin-resistant staphylococci in Lithuanian horse nasal mucosa was detected (10 strains). Also we detected staphylococci which showed resistance to penicillin, tetracycline or erythromycin. These antimicrobials were registered for the treatment of horses in Lithuania long time ago and is most frequently conformable in veterinary preparations (http://vetlt1.vet.lt/vr/). The same findings were made in other countries, where (Haeni et al., 2010) resistance of *S. aureus* to antibiotics of medical and/or veterinary interest is big. The highest proportions of resistance were observed for penicillin (62.7 %), tetracycline (23.7 %), and the aminosides (approximately 10%). The mentioned researchers also reported that in their research *S. pseudintermedius* isolate presented resistance to penicillin, streptomycin and kanamycin and was devoid of the *mec*A gene. The same was confirmed in our investigation, where the majority CNS and CPS was resistant to many antimicrobic drugs, even not having resistance genes. *Mec*A gene was determined only at six CNS izoliates, two *tet*K, one *tet*M and two *S. aureus* strains with *bla*Z gene. Meanwhile, in *S. aureus* isolates we have not registered *mec*A gene. Isolates with *Mec*A gene were obtained from four healthy horses and two horses which were under treatment in large animals clinics. Two horses, having CPS with m*ec*A gene are held in big stud farm, but their stalls are in different pavilions of stud. This fact matches the hypothesis foreign of researchers (Corrente et al., 2009) that MRCNS of people and MRCNS of horses is one reservoir. Apparently, in this case the stableman, rider or owner of the horse were mediators transferring the resistance gene. In addition, isolate contained the *mec*A gene and had a high resistance to β- lactam antibiotics. These isolates on MRSA agar grew in blue colonies, which means, that their MIC for penicillin is higher than 2 mg/L. Six isolates extracted in our research had penicillin MIC over 0.25 mg/l what shows that all of them were producing beta lactamases. Their MIC to oxacillin also was 4–8 mg/L. According to EUCAST, all these isolates should have *mec*A gene, which was indicated in our research. According to Yasuda et al. (2000), some isolates also often were resistant to other antibiotics, such as erythromycin and kanamycin. Yet this was not proved in our research. The isolates, in our research, did not have additional genes, such as resistance genes to aminoglycosides (*aac(6')-Ie-aph(2')-Ia, aph(3')-III*, *aph(2')-Ic)* and macrolides (*mrs, erm*B and *erm*C, tetracycline *tet*K and *tet*M). In our research, the resistance to β-lactam antibiotics resulted mainly from the presence of *bla*Z or *mec*A genes. Two more CPS isolates extracted from the horse nasal mucosa had *bla*Z gene. These strains carrying *bla*Z were resistant to penicillin and produced a β-lactamase. Resistance to tetracycline attributed to the efflux gene *tet*K and, to a lesser extent, to ribosomal protection encoded by the *tet*M gene. They were identified in three strains. Their MIC was over 16 mg/L.

As it is, we think, that methicillin-resistant CNS, which were isolated from healthy horses in Lithuania, is a potential threat for horses and veterinarians who are taking care of such horses.

Conclusions. The highest resistance rate among all staphylococci horse nasal mucosa isolates were *S. aureus* (42.9 %), *S. lentus* (14.3 %), *S. pseudintermedius*

(13.1 %), *S. cohnii* and *S. xylosus* (9.5 %). *S. sciuri, S. warneri*, and *S. vitulinus* were isolated at low rates.

Staphylococci resistance rates were highest to penicillin G (66.7 %), erythromycin (41.6 %) and tetracycline (33.2 %). All isolates were susceptible to vancomycin, daptomycin and trimethoprim/sulfamethoxazole.

Among ten methicillin resistant isolates *mec*A gene was found in six of them: 2 strains *S. lentus*, *S. sciuri* and *S. vitulinus*. Presence of *mec*C gene were not detected. *Bla*Z gene was detected in two *S. aureus* isolates.

All isolates were resistant to macrolides and aminoglycosides, but resistance genes *aac*, *aph*, *msr*A/B, *erm*A, and *erm*C in these strains were not detected. Resistance to tetracycline was encoded by *tet*K and *tet*M genes only.

Acknowledgement. This research was funded by a grant (MIP-061/2012) from the Research Council of Lithuania.

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Received 22 September 2014
Accepted 30 October 2014