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Identification and Methicillin Resistance of Coagulase-Negative Staphylococci Isolated from Nasal Cavity of Healthy Horses

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The aim of this study was an analysis of the staphylococcal flora of the nasal cavity of 42 healthy horses from 4 farms, along with species identification of CoNS isolates and determination of resistance to 18 antimicrobial agents, particularly phenotypic and genotypic methicillin resistance. From the 81 swabs, 87 staphylococci were isolated. All isolates possessed the *gap* gene but the *coa* gene was not detected in any of these isolates. Using PCR-RFLP of the *gap* gene, 82.8% of CoNS were identified: *S. equorum* (14.9%), *S. warneri* (14.9%), *S. sciuri* (12.6%), *S. vitulinus* (12.6%), *S. xylosus* (11.5%), *S. felis* (5.7%), *S. haemolyticus* (3.4%), *S. simulans* (3.4%), *S. capitis* (1.1%), *S. chromogenes* (1.1%), and *S. cohnii* subsp. *urealyticus* (1.1%). To our knowledge, this was the first isolation of *S. felis* from a horse. The species identity of the remaining *Staphylococcus* spp. isolates (17.2%) could not be determined from the *gap* gene PCR-RFLP analysis and 16S rRNA gene sequencing data. Based on 16S-23S intergenic transcribed spacer PCR, 11 different ITS-PCR profiles were identified for the 87 analyzed isolates. Results of API Staph were consistent with molecular identification of 17 (19.5%) isolates. Resistance was detected to only 1 or 2 of the 18 antimicrobial agents tested in the 17.2% CoNS isolates, including 6.9% MRCoNS. The *mecA* gene was detected in each of the 5 (5.7%) phenotypically cefoxitin-resistant isolates and in 12 (13.8%) isolates susceptible to cefoxitin. In total, from 12 horses (28.6%), 17 (19.5%) MRCoNS were isolated. The highest percentage of MRCoNS was noted among *S. sciuri* isolates (100%).

Keywords: coagulase-negative staphylococci, horse, *gap* gene, *mecA* gene, methicillin, antimicrobial agents

Introduction

The genus *Staphylococcus* is divided into 42 validly described species and subspecies, 10 of which contain subdivisions with subspecies designations (Ghebremedhin *et al.*, 2008). The majority of species belong to coagulase-negative staphylococci (CoNS) (Layer *et al.*, 2007). Numerous phenotypic and genotypic methods are used to identify the species of staphylococci. However, methods based on phenotypic properties are dependent on the expression of metabolic activity and/or morphological features, which are often difficult or impossible to identify. Currently, due to the development of molecular biology techniques, DNA is often used in microbiology as a tool for classification of microorganisms (Becker *et al.*, 2004; Harmsen and Karch, 2004).

It can be assumed, that members of the genus *Staphylococcus* are the most important bacteria among the natural microflora of the skin and mucous membranes. Coagulase-negative staphylococci (CoNS) are a diverse group of commensals inhabiting the skin and mucous membranes of humans and animals, however some species are known as important opportunistic human pathogens. The role of CoNS as animal pathogens is less understood. Some of the CoNS species are involved in diseases of various animals, including wound, respiratory system, eye and uterine infections in horses (Moodley and Guardabassi, 2009). The current knowledge regarding the occurrence, differentiation and antibiotic resistance of staphylococci isolated from animals is mainly based on clinical isolates, but little is known about commensal staphylococci colonizing the nasal cavity (Bagcigil *et al.*, 2007).

Methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) are a serious problem for human and animal populations (De Martino *et al.*, 2010; Huber *et al.*, 2011). Resistance to methicillin is determined by the *mecA* gene, located on a large mobile genetic element defined as the staphylococcal cassette chromosome *mec* (SCC*mec*). It is speculated that SCC*mec* is transmissible across staphylococcal species (Tsubakishita *et al.*, 2010; Huber *et al.*, 2011). Currently, 7 major variants of SCC*mec* (types I-VII) are distinguished (Higuchi *et al.*, 2008; De Martino *et al.*, 2010). Resistance to methicillin is more common among CoNS than *S. aureus* (Moodley and Guardabassi, 2009). Methicillin-resistant staphylococci (MRS) are present in the horse population and may be a reservoir of new or rare strains of MRSA and MRCoNS (Baptiste *et al.*, 2005; De Martino *et al.*, 2010). MRCoNS are isolated from various domesticated, healthy animals (Huber *et al.*, 2011), including the nasal cavity of horses (Yasuda *et al.*, 2000, 2002; Bagcigil *et al.*, 2007; De Martino *et al.*, 2010). Although the role of nasal carriage in *S. aureus* infections in humans is known well, it remains unclear in animals. Moreover, the importance of carriage of CoNS in the nose,

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including MRCoNS, both in humans and animals, has not been elucidated (De Martino *et al*., 2010).

The aim of this study was the analysis of staphylococcal flora inhabiting the nasal cavity of healthy horses, identification of CoNS species and determination of antibiotic resistance, particularly genotypic and phenotypic resistance to methicillin.

Materials and Methods

Sampling

Swabs were taken from the nasal cavities of 42 riding horses of the Polish noble half-bred breed, in the period from July to August 2009. Horses came from four farms (labeled A-D) from north-western Poland: A (12 horses), B (10 horses), C (12 horses), and D (8 horses). Horses aged 8 to 15 years, were clinically healthy and had not been treated with antibiotics within 3 months of sample collection. Swabs were taken from both nostrils of each horse. Sterile swabs were introduced separately into each nostril to a depth of 10 cm and rolled on the mucosal membranes for 5 sec after carefully cleaning the front of the nostrils and nasal mucous membrane with a disinfectant. Swabs were transported to the laboratory in Stuart's medium (Oxoid, UK) at 4°C and analyzed within 24 h.

Isolation and phenotypic identification

Each swab was inoculated in parallel on Mannitol Salt Agar medium (Oxoid), CHROMagar *Staphylococcus aureus* medium (Graso, Poland) selective for *S. aureus* and CHROMagar MRSA medium (Graso) selective for MRSA, and incubated aerobically at 37°C for 24–48 h. *S. aureus* ATCC (American Type Culture Collection) 25923 (MSSA) and *S. aureus* ATCC 43300 (MRSA) were used as control strains. Isolates were Gram stained and catalase activity and capacity for aerobic and anaerobic glucose fermentation were determined. The ability to produce coagulase and presence of clumping factor (CF) were determined using rabbit plasma (Biomed, Poland). The API Staph test (bioMérieux, France) was used for biochemical identification. Identification was categorized into one of four confidence levels: excellent (%ID ≥ 99.9, T index ≥ 0.75), very good (%ID ≥ 99.0, T index ≥ 0.5), good (%ID ≥ 90.0, T index ≥ 0.25), and acceptable (%ID ≥ 80, T index ≥ 0). Results below these levels were categorized as unacceptable.

PCR

DNA was isolated using the Genomic Mini kit (A&A Biotechnology, Poland). Fragments of the *gap* gene, *coa* gene, *mecA* gene, 16S–23S rRNA intergenic transcribed spacer region and 16S rRNA gene were amplified using primers and PCR conditions (with modifications) previously described by Yugueros *et al.* (2000), da Silva and da Silva (2005), Oliveira and de Lencastre (2002), Mendoza *et al.* (1998), and Young-Duck *et al.* (2007), respectively. The PCR was performed in a final volume of 25 µl containing: 0.7 U AmpliTaq Gold DNA polymerase 360 (5 U/µl) (Applied Biosystems, USA), 10× polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.8 µM of each primer for the *gap* gene and *mecA* gene, 0.5 µM of each primer for the *coa* gene, the 16S–23S rRNA intergenic transcribed spacer region and 16S rRNA gene, approximately 10 ng DNA. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, 40 cycles for the *gap* gene, 30 cycles for the *coa* gene, *mecA* gene and 16S rRNA gene and 25 cycles for the 16S–23S rRNA intergenic transcribed spacer region: DNA denaturation at 95°C for 30 sec, annealing of primers for 30 sec in 50°C (*gap* gene, 16S–23S rDNA intergenic region), 55°C (*mecA* gene, 16S rRNA gene) or 65°C (*coa* gene), extension at 72°C for 1 min; final extension at 72°C for 7 min.

PCR-RFLP

The *gap* gene amplification products were digested with *Alu*I restriction enzyme (Fermentas, USA) according to the manufacturer's recommendations.

Electrophoresis

PCR products were separated on 2% agarose gels (peqGOLD, Peqlab, Germany) in 1× TBE buffer (Bio-Rad, USA), visualized by staining with 1% aqueous solution of ethidium bromide (Merck, Germany) and analyzed using GenTools software (Syngene, UK).

In silico analysis of *gap* gene polymorphism

In silico analysis after digestion with *Alu*I enzyme (NEBcutter V2.0) was performed for the sequences of the *gap* gene of 55 species and subspecies of staphylococci, deposited in GenBank (NCBI database, search dated June 9, 2011). The generated *in silico* RFLP profiles were used as species standards for interpreting the results of PCR-RFLP of the *gap* gene, obtained for the investigated isolates.

16S rRNA gene sequencing

Sequencing was performed by the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (IBB PAN, Poland). The sequenced 240 bp-long fragment of the 16S rRNA gene of *Staphylococcus* spp. was deposited in GenBank under accession number JN654462. Sequencing analysis was carried out using the BLAST program, based on the GenBank nucleotide database (search dated 8 August 2011) at the National Center for Biotechnology Information (NCBI).

Antimicrobial susceptibility testing

Susceptibility of staphylococci to 18 antimicrobial agents was assessed by the disc-diffusion method, in accordance with the standards of Clinical and Laboratory Standards Institute (CLSI, 2008). As a control, strains *S. aureus* ATCC 25923 (MSSA) and *S. aureus* ATCC 43300 (MRSA) were used. The following antibiotic discs were used (Oxoid): penicillin G (10 U), cefoxitin (30 µg), amoxicillin/clavulanic acid (30 µg), cefuroxime (30 µg), cephalothin (30 µg), imipenem (10 µg), gentamicin (10 µg), kanamycin (30 µg), erythromycin (15 µg), clindamycin (2 µg), quinupristin/dalfopristin (15 µg), tetracycline (30 µg), doxycycline (30 µg), enrofloxacin (5 µg), sulfamethoxazole/trimethoprim (23.75+1.25 µg), chloramphenicol (30 µg), rifampicin (30 µg), linezolid (30 µg).

Table 1. **Isolation and prevalence of staphylococcal species on four horse farms**

Horse farm	No. of horses	No. of swabs	No. of isolates	*Staphylococcus* species / group
A	12	23	25	*S. vitulinus* n=7 *Staphylococcus* spp. n=6 *S. equorum* n=4 *S. simulans* n=3 *S. xylosus* n=3 *S. capitis* n=1 *S. warneri* n=1
B	10	19	23	*Staphylococcus* spp. n=9 *S. equorum* n=5 *S. felis* n=2 *S. vitulinus* n=2 *S. warneri* n=2 *S. cohnii* subsp. *urealyticus* n=1 *S. haemolyticus* n=1 *S. xylosus* n=1
C	12	23	23	*S. warneri* n=10 *S. felis* n=3 *S. sciuri* n=3 *S. equorum* n=2 *S. haemolyticus* n=2 *S. xylosus* n=2 *S. chromogenes* n=1
D	8	16	16	*S. sciuri* n=8 *S. xylosus* n=4 *S. equorum* n=2 *S. vitulinus* n=2
Total	42	81	87	

The prevalence of macrolide–lincosamide–streptogramin B (MLS_B) resistance was investigated by the double-disk diffusion test with erythromycin (15 µg) and clindamycin (2 µg) (Fiebelkorn *et al.*, 2003).

Results

On the basis of the growth on chromogenic media, *S. aureus* and MRSA were not detected, in any of the swabs. For

Table 2. **The *gap* gene-based identification, API-based identification and antimicrobial resistance of staphylococcal isolates**

Staphylococcus species / group[a]	No. of isolates (%)	API Staph (%ID ; *T* index[b])	Phenotype of resistance (No. of isolates)	*mecA*	Total of *mec-A* isolates
Staphylococcus spp.	15 (17.2)	*S. schleiferi* (94.4 ; 0,4) *S. xylosus* (82.9 ; 0.5)	TE (1)	-	1
S. equorum	13 (14.9)	non typeable	P (1), E (1), P-E (1), P-AMC (2)	- - - -	0
S. warneri	13 (14.9)	*S. hominis* (59.0 ; 0.4)	P (1)	-	0
S. sciuri	11 (12.6)	*S. xylosus* (92.2 ; 0.5) *S. sciuri* (69.9 ; 0.6)	P-FOX (3)	+	11
S. vitulinus	11 (12.6)	*S. xylosus* (82.4 ; 0.4)	P-FOX (1), P-E (1)	+ +	4
S. xylosus	10 (11.5)	*S. xylosus* (99.8 ; 0.7)	CN (1), P-FOX (1)	- +	1
S. felis	5 (5.7)	*S. sciuri* (93.8 ; 0.7)		-	0
S. haemolyticus	3 (3.4)	*S. haemolyticus* (91.0 ; 0.4)		-	0
S. simulans	3 (3.4)	*S. simulans* (85.0 ; 0.3)		-	0
S. capitis	1 (1.1)	nontypeable	P (1)	-	0
S. chromogenes	1 (1.1)	*S. chromogenes* (82.0 ; 0.3)		-	0
S. cohnii urealyticus	1 (1.1)	*S. hominis* (78.8 ; 0.6)		-	0
	87		15 (17.2%)	6 (6.9%)	17 (19.5%)

[a] Identification according to *gap* gene PCR-RFLP profiles
[b] %ID – percentage of identification (average); *T* index (average)
P, penicillin (10 U); FOX, cefoxitin (30 µg); AMC, amoxicillin/clavulanic acid (30 µg); CN, gentamicin (10 µg); E, erythromycin (15 µg); TE, tetracycline (30 µg)

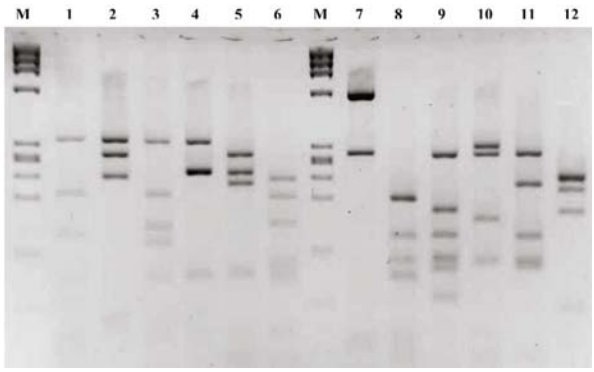

Fig. 1. Agarose gel electrophoresis of the PCR-RFLP products of *gap* gene of analyzed groups of staphylococcal species. Lanes: M, ΦX174 DNA/*Bsu*RI (*Hae*III) (Fermentas); 1, *S. capitis*; 2, *S. chromogenes*; 3, *S. equorum*; 4, *S. haemolyticus*; 5, *S. cohnii* subsp. *urealyticus*; 6, *S. vitulinus*; 7, *S. felis*; 8, *S. sciuri*; 9, *S. simulans*; 10, *S. warneri*; 11, *S. xylosus*; 12, *Staphylococcus* spp.

further study, morphologically different colonies grown on Mannitol Salt Agar were chosen. From three swabs, staphylococci were not isolated. Most of the isolates (80%) fermented mannitol on Mannitol Salt Agar medium. In total, from 81 swabs of 42 horses 87 Gram-positive and catalase-positive cocci were isolated (Table 1). None of the isolates produced coagulase or had clumping factor. From 34 horses – 2 isolates, from 4 horses – 3 isolates, from 3 horses – 1 isolate and from 1 horse – 4 isolates of staphylococci were obtained. From 25 horses – 1 species, from 14 horses – 2 species and from 3 horses – 3 species of staphylococci were identified. All of the 87 isolates possessed the *gap* gene (933 bp), a molecular marker for *Staphylococcus*; however, the *coa* gene was not detected, confirming the absence of *S. aureus* among the isolates. Based on the PCR-RFLP with *Alu*I restriction enzyme, 12 groups of species of staphylococci (Table 2), characterized by distinct restriction patterns of the *gap* gene (Fig. 1) were separated. In total, 72 (82.8%) isolates of staphylococci were identified. Isolates of *S. equorum*, *S. warneri*, *S. sciuri*, *S. vitulinus*, *S. xylosus*, *S. felis*, *S. haemolyticus*, *S. simulans*, *S. capitis*, *S. chromogenes*, and *S. cohnii* subsp. *urealyticus* were identified precisely. The results were confirmed by the *gap* gene RFLP *in silico* analysis with *AluI* restriction enzyme using the *gap* gene sequences deposited in GenBank under the following accession numbers: DQ321684, AY024363, DQ321697, EU659916, DQ321700, DQ321685, DQ321687, DQ321698, DQ321676, DQ321680, HM352971, respectively. However, the high homology, ranging from 97–99% (ClustalW2 program) within the *gap* gene sequences of *S. equorum* and subspecies (*S. equorum equorum* – EU659907, *S. equorum linens* – HM352977), *S. sciuri* and subspecies (*S. sciuri sciuri* – FJ578000, *S. sciuri carnaticus* – FJ578001, *S. sciuri rodentium* – HM352984) and *S. capitis* and subspecies (*S. capitis capitis* – EU659902, *S. capitis urealyticus* – HM352966), made further intraspecific differentiation impossible. For the remaining 15 (17.2%) isolates of *Staphylococcus* spp. a common, but unidentifiable, *gap* gene PCR-RFLP pattern was obtained. The *gap* gene sequence with this polymorphism does not exist in the GenBank database, making it impossible to determine if those *Staphylococcus* spp. isolates belong to a particular species. Also, *in silico* analysis (BLAST program) of the sequenced fragment of 16S rRNA did not permit unambiguous species identification (*S. auricularis*, *S. pasteuri*, *S. sciuri*, *S. warneri*, *S. lugdunensis*, *Staphylococcus* spp.) of the unidentified staphylococci. Moreover, *in silico* analysis of RFLP patterns of the *gap* gene (NEBcutter V2.0) for the BLAST-indicated species (*S. auricularis* – GenBank accession no. AF495476, *S. pasteuri* – HM352972, *S. sciuri* – AF495496, *S. warneri* – DQ321699, *S. lugdunensis* – DQ321693) precludes the assignment of the investigated *Staphylococcus* spp. to any of those species.

Based on the analysis of the 16S-23S rRNA intergenic transcribed spacer region (ITS-PCR) 11 different profiles, consisting of 4 to 5 PCR products ranging in length from approximately 300–600 bp were identified (data not shown). For isolates of *Staphylococcus* spp., *S. equorum*, *S. warneri*, *S. chromogenes*, *S. haemolyticus*, *S. simulans*, *S. capitis*, *S. cohnii* subsp. *urealyticus* and for one of the 10 isolates of *S. xylosus* different ITS-PCR profiles were obtained. In turn, a different profile was recorded for nine other isolates of *S. xylosus* and for all isolates of *S. felis*, and another one for all isolates of *S. sciuri* and *S. vitulinus*.

The results from API Staph (Table 2) were consistent with the results of molecular identification for 17 (19.5%) isolates, representing four species: *S. xylosus* (very good identification), *S. haemolyticus* (good identification), *S. simulans*, and *S. chromogenes* (acceptable identification). In turn, biochemical patterns obtained for 14 (16.1%) isolates of *S. equorum* and *S. capitis* do not exist in the APIweb database. Among 15 (17.2%) isolates of *Staphylococcus* spp., the API system identified *S. schleiferi* and *S. xylosus*, but the *gap* gene PCR-RFLP profiles of these isolates exclude their belonging to any of these species. The remaining 41 (47.1%) isolates were identified incorrectly (*S. warneri*, *S. vitulinus*, *S. felis*, *S. cohnii* subsp. *urealyticus*) or the %ID was too low (*S. sciuri*).

Among the 87 isolates, 5 (5.7%) were resistant to penicillin G and cefoxitin, 3 (3.4%) to penicillin G, 2 (2.3%) to penicillin G and amoxicillin/clavulanic acid, 2 (2.3%) to penicillin G and erythromycin, 1 (1.1%) to erythromycin, 1 (1.1%) to gentamicin and 1 (1.1%) to tetracycline (Table 2). The *mecA* gene was detected in all 5 cefoxitin-resistant isolates. Furthermore, the *mecA* gene was found in 12 cefoxitin-susceptible isolates: *S. sciuri* (8 isolates), *S. vitulinus* (3 isolates), and *Staphylococcus* spp. (1 isolate) (Tables 2–3). In total, 17 (19.5%) *mecA*-positive staphylococci (*S. sciuri* – 11 isolates, *S. vitulinus* – 4 isolates, *S. xylosus* – 1 isolate, *Staphylococcus* spp. – 1 isolate) were identified among 12 horses (28.6%). The highest percentage of *mecA*-positive staphylococci were identified in horses of farm D (68.8%), followed by C (13%), A (8%), and B (4.3%) (Table 3). On the basis of simultaneous erythromycin resistance and susceptibility to clindamycin, in two isolates of *S. equorum*, and in one *S. vitulinus*, a MS_B resistance phenotype was identified (Table 2). However, among all the staphylococci, constitutive or inducible resistance to macrolides, lincosamides and streptogramin B (MLS_B phenotype) was not found.

Table 3. The prevalence of methicillin resistant CoNS among the horse farms

Horse farm	Total isolates	No. of horses	*Staphylococcus* species / group	No. of *mecA*(+) isolates	Total *mecA*(+) isolates (%)	Resistance to cefoxitin (No. of isolates)
A	25	1	*S. vitulinus*	1	2 (8)	-
		1	*S. vitulinus*	1		-
B	23	1	*Staphylococcus* spp.	1	1 (4.3)	-
C	23	1	*S. sciuri*	2*	3 (13)	+ (1)
		1	*S. sciuri*	1		+ (1)
D	16	1	*S. sciuri*	2*	11 (68.8)	-
		1	*S. sciuri*	2*		-
		1	*S. sciuri*	1		+ (1)
		1	*S. sciuri*	1		-
		1	*S. vitulinus*	1		+ (1)
		1	*S. sciuri*, *S. vitulinus*	2*		-
		1	*S. sciuri*, *S. xylosus*	2*		- , + (1)
	87	12 (28.6 %)			17 (19.5%)	5 (5.7%)

* each isolate from the other nostril

Discussion

For the genotypic identification of staphylococci and classification of the species, various markers can be used, for example, 16S rRNA gene, 16S–23S rRNA intergenic transcribed spacer (ITS), *hsp60* gene, *femA* gene, *sodA* gene, *tuf* gene, *rpoB* gene or *gap* gene (Ghebremedhin *et al*., 2008). According to Yugueros *et al.* (2000), Layer *et al.* (2007), and Ghebremedhin *et al.* (2008), polymorphic sequences of the *gap* gene are a good tool for differentiation of staphylococci, and allow the identification of 28 species within the genus. In this report, based on *gap* gene polymorphism, 82.8% of isolates belonging to 11 species and/or subspecies were identified. 17.2% of isolates (*Staphylococcus* spp.) with the same PCR-RFLP profile for the *gap* gene could not be identified. Among the available data for *gap* gene sequences in the GenBank database, for 55 species of staphylococci, a sequence yielding such a PCR-RFLP profile does not exist. Moreover, PCR-RFLP profiles of the *gap* gene of *S. equorum*, *S. sciuri*, and *S. capitis*, analyzed with sequences available through GenBank, did not allow for intraspecific differentiation. The deposited sequences showed high homology and polymorphism unidentifiable by the PCR-RFLP technique. Currently, identification of the CoNS is often undertaken by the methods based on sequencing of PCR amplicons, e.g., the 16S rRNA, *sodA*, and *tuf* genes (Heikens *et al*., 2005; Ghebremedhin *et al*., 2008). Analysis of the 16S rRNA gene is recommended as a useful and extensively investigated taxonomic marker (Becker *et al*., 2004; Harmsen and Karch, 2004). However, in this case, amplification and sequencing of 16S rRNA gene also failed to identify *Staphylococcus* spp. isolates. The alignment result obtained by BLAST for the analyzed sequence of the 16S rRNA gene was not clear. Moreover, the RFLP profile of the *gap* gene of species indicated by BLAST, did not allow the assignment of the investigated *Staphylococcus* spp. isolates to any of those species. It can be assumed that these isolates belong to a new, not yet described species within the genus *Staphylococcus*. This, however, requires further study.

Some authors point out that a disadvantage of the sequence-based, genotypic method is that it suffers from the poor quality of some sequences deposited in databases, especially the 16S rRNA gene sequences deposited in GenBank (Heikens *et al*., 2005). The Ribosomal Differentiation of Microorganisms (RIDOM) database has been proposed as an alternative database (Harmsen *et al*., 2002; Becker *et al*., 2004). Becker *et al.* (2004) analyzed the 16S rRNA-derived results using the RIDOM database in comparison to the entries of the NCBI sequence database. Using the GenBank database at NCBI, the authors could not identify 33% of the isolates of staphylococci, including *S. aureus*, whereas using the RIDOM database, ambiguous results were obtained for only 7% of the isolates.

Amplification of the 16S–23S rRNA intergenic transcribed spacer region (ITS-PCR) is also considered to be a good tool for intra-species differentiation of staphylococci (Couto *et al*., 2001; Fujita *et al*., 2005). In this study, based on the analysis of ITS-PCR products, 11 different profiles were identified. For eight groups of staphylococci, species specific and distinct patterns were obtained. However, the same ITS-PCR profile was returned for 2 species and 2 different profiles were found for one species (one of which was common for two species). The results did not confirm that the 16S–23S rRNA intergenic transcribed spacer regions are a good source of species-specific sequences (Couto *et al*., 2001; Nováková *et al*., 2006). In turn, the results confirmed that the strains of one species may exhibit polymorphism in the 16S-23S rRNA intergenic region (Gürtler and Stanisich, 1996), as was found in *S. xylosus* isolates. The problems with the identification of certain species of staphylococci by the ITS-PCR method were also noted by Couto *et al.* (2001).

To our knowledge, this is the first isolation of *S. felis* from horses. *S. felis* was first isolated from a number of clinical samples of felines and described by Igimi *et al.* (1989) as coagulase-negative staphylococcus, phenotypically similar to *S. simulans*. In this study, isolates of *S. felis* were identified by API Staph as *S. sciuri*. Biochemical similarity of *S. felis* to other than *S. simulans* species of staphylococci was also confirmed by Becker *et al.* (2004). There are reports of methicillin-resistant *S. felis* isolated from the skin of clinically healthy cats (Lilenbaum *et al*., 1998). However, in our research none of the five *S. felis* isolates showed phenotypic or genotypic resistance to methicillin. The presence of *S. felis* in horses, including possible methicillin resistant isolates of this species, seems to be an interesting issue that

needs further study.

Commercial tests for the identification of staphylococci, based on biochemical reactions, such as API Staph, often do not allow for the identification of CoNS species, especially those isolated from animals, because of the limited number of animal strains in their databases (Onni *et al.*, 2010). In our study, the API system correctly identified 19.5% of isolates. For the remaining 80.5% of isolates identification was incorrect or was characterized by very low probability, or the obtained biochemical profile was not recognized by APIweb at all (Table 2). It should be noted that *S. equorum*, *S. vitulinus*, and *S. felis* do not exist in the APIweb database. That explains the failure to biochemically identify these species in our study. Busscher *et al.* (2006), Sampimon *et al.* (2009), and Onni *et al.* (2010) reported similar problems with identification of CoNS isolated from animals using the API system. Moreover, the problem of biochemical identification, using the API Staph system, also concerns the reference strains as well as clinical isolates from humans, as was confirmed by Renneberg *et al.* (1995).

Methicillin-resistant coagulase-negative staphylococci (MRCoNS) and methicillin-resistant *S. aureus* colonize the mucous membranes of various animal species, however the importance of carriage of staphylococci in the nose of the animals has not been elucidated (Yasuda *et al.*, 2000; Bagcigil *et al.*, 2007). Colonization with MRSA in horses ranges from 0–16% (Baptiste *et al.*, 2005; Busscher *et al.*, 2006; Burton *et al.*, 2008; Moodley and Guardabassi, 2009). Burton *et al.* (2008) did not isolate MRSA from any of 497 healthy horses. The results obtained in this study confirm the low rate of carriage of MRSA in healthy horses.

The prevalence of coagulase-negative staphylococci, including MRCoNS, in the nasal cavity of healthy horses, as was found in our research, was consistent with previous reports from Yasuda *et al.* (2000), Busscher *et al.* (2006), Moodley and Guardabassi (2009), De Martino *et al.* (2010). In our study, CoNS were isolated from the nasal cavity of all 42 healthy horses. Additionally, in 12 (28.6%) horses *mecA*-positive isolates belonging to four species were found (Table 3). The highest percentage of MRCoNS was found among *S. sciuri* (100%), followed by *S. vitulinus* (36%), *S. xylosus* (10%), and *Staphylococcus* spp. (7%) (Table 2). The results are consistent with the report by De Martino *et al.* (2010). They isolated 68 *mecA*-positive staphylococci from the nasal cavity of 35% of healthy horses from 3 farms, including 66 CoNS isolates. The highest percentage of MRCoNS was found among *S. sciuri* species. In one of the three farms they found 54% of MRS, the percentages in two other farms was 22% and 24%. The differences in distribution of MRS might be connected with frequency of antibiotic use, the environment, physical activity, age or stress. In this work, most of the MRCoNS were found in horses from farm D, followed by C, A, and B (Table 3). However, horses on all farms were maintained under similar conditions and were free of antibiotic therapy for 3 months prior to taking the swab. The only differentiating factor was the age of the horses (8 to 15 years), however, no correlation between age and the occurrence of MRCoNS was found in these horses. Further, Busscher *et al.* (2006), isolated CoNS phenotypically resistant to methicillin from 57% of healthy horses (swab samples were taken from nostrils and fetlocks). However *mecA*-positive staphylococci were isolated from just 23% of horses. In total, Busscher *et al.* (2006) identified 81% of *mecA*-positive CoNS, the predominant species was *S. sciuri*. In this study, *mecA*-positive staphylococci were isolated from 28.6% (12/42) of horses, but isolates phenotypically resistant to methicillin were found in only 11.9% (5/42) of horses (Table 3). In contrast, Bagcigil *et al.* (2007), isolated MRCoNS from 50% of horses from an equine farm and an animal hospital. The most frequent species was *S. vitulinus*, followed by *S. haemolyticus*, *S. sciuri*, and *S. epidermidis*. It was the first isolation of *mecA*-mediated methicillin resistant *S. vitulinus*. This species is considered to be part of the commensal flora of horses and other animals, rarely associated with infections in animals and humans. Moodley and Guardabassi (2009), isolated MRCoNS from the nasal cavity of 82% of healthy and sick horses, among which *S. vitulinus* predominated, followed by *S. sciuri*, *S. haemolyticus*, and *S. equorum*. In contrast to the results of Bagcigil *et al.* (2007) and Moodley and Guardabassi (2009), *S. haemolyticus* and *S. equorum* isolated in our research were *mecA*-negative and among MRS, *S. sciuri* followed by *S. vitulinus* were most prevalent (Table 3). The percentage of *mecA*-positive isolates of *S. sciuri* and *S. vitulinus* was respectively 100% and 36%, which corresponds with the results (100% and 30% respectively) obtained by Tsubakishita *et al.* (2010).

Our findings confirm the widespread presence of the determinants of methicillin resistance among *S. sciuri* (100% *mecA*-positive isolates), although phenotypic methicillin resistance was shown only for 3 (27.3%) isolates of this species (Tables 2 and 3). The reservoir of the *mecA* gene has remained unclear. It is speculated that the origin of the *mecA* gene would be found in staphylococcal species closely associated with *S. sciuri* and *S. vitulinus* that belong, together with *S. lentus* and *S. fleuretti,* to the *S. sciuri* group (Fuda *et al.*, 2007; Tsubakishita *et al.*, 2010; Huber *et al.*, 2011). Couto *et al.* (2001) and Fuda *et al.* (2007) reported that *S. sciuri* can accumulate resistance genes for several classes of antibiotics such as penicillin and clindamycin. In our study, only 3 (27%) of the *S. sciuri* isolates were resistant to penicillin and cefoxitin, showing sensitivity to other antimicrobial agents, including clindamycin.

Due to the frequent use of β-lactam antibiotics and macrolides in animals, in particular to treat staphylococcal infections, it is important to monitor the resistance of staphylococci to these groups of drugs. Bagcigil *et al.* (2007) in studies of MRCoNS isolated from the nasal cavity of horses that were treated with antibiotics, found resistance to a wide range of antibiotics commonly used to treat staphylococcal infections, including β-lactams, ciprofloxacin, quinupristin/dalfopristin. In our study, CoNS isolated from healthy horses were susceptible to most antimicrobial agents, which confirms the key role of antimicrobial pressure in the spread of drug resistance. In total, 15 (17.2%) isolates of CoNS, including 6 (6.9%) MRCoNS were resistant only to 1 or 2 antimicrobial agents: β-lactams, erythromycin, gentamicin and/or tetracycline. The results are consistent with previous research conducted by Busscher *et al.* (2006) who, in 98% of *mecA*-positive CoNS isolated from healthy horses, showed sensitivity to most of the antibiotics tested, and in

only 2% found multidrug resistance.

To summarize, it was shown that the PCR-RFLP of the *gap* gene is a more effective and discriminative method for identifying species of staphylococci, than the analysis of the 16S–23S rRNA intergenic transcribed spacer region or biochemical identification. In addition, colonization of *S. aureus* in the nasal cavity of healthy horses was not found, which confirms the minor importance of carrying *S. aureus* and MRSA for this population of animals. However colonization with different species of CoNS including MRCoNS was detected, which can function as a protective factor against pathogens. It has previously been shown that coagulase-negative staphylococci produce inhibitory substances acting antagonistically against pathogenic species (Matthews *et al.*, 1991).

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