METHICILIN-RESISTANT \textit{STAPHYLOCOCCUS XYLOSUS} ISOLATED FROM HORSES AND THEIR SENSITIVITY TO ENTEROCINS AND HERBAL SUBSTANCES

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ABSTRACT

Methicillin-resistant (MR) strains \textit{S. xylosus} from faeces of horses were tested for their sensitivity to enterocins (\textit{Ents}) and herbal substances (oregano, sage) to contribute to basic research related to \textit{Ents} and horses microbiota. The counts of staphylococci in faecal samples of 122 horses reached log10 2.03±0.42 colony forming units per gram. Twelve staphylococcal isolates were found to be MR and they were taxonomically allotted to the species \textit{S. xylosus}. The strains produced lactic acid at average of 1.04±0.004 mmol/l. Six strains were not sensitive to \textit{Ents} used; however, they were sensitive to oregano and sage substances reaching inhibitory zones in size of 17-31 mm. The other strains were sensitive at least to 3 of 5 \textit{Ents} tested (inhibitory activity 100 - 12 800 AU/ml) and they were also sensitive to both herbal substances. In general, the growth of the strains \textit{S. xylosus} was inhibited by oregano and sage substances; however, the inhibitory zones due to oregano possessed 28 mm in average and due to sage 12 mm in average. The strains were less sensitive to \textit{Ents} than to herbs.

Key words: horses; \textit{Staphylococcus xylosus}; enterocins; oregano; sage; sensitivity

INTRODUCTION

In general, microbiology of the equine gastrointestinal tract is poorly characterized. Garret \textit{et al.} (2002) detected Gram-positive cocci in the faeces of healthy horses at the amount of 10^8 colony forming units (cfu per gram, mean count) and 10^6 cfu/g of Gram-negative rods. Staphylococci belong to the phylum Firmicutes, to the genus Staphylococcus and to the Family Staphylococcaceae (Bergeys Manual, 2009). The counts of staphylococci in faecal samples from farm horses reached about log10 3.0 cfu/g (unpublished results). From the medical point of view, methicillin-resistant (MR) staphylococci belong to one of the most serious therapeutic problems. Resistance to methicillin in staphylococci appeared as the result of production of a novel penicillin-binding protein (PBP)-PBP2a with a low affinity for beta-lactam antibiotics (Chamber, 1997). In the Netherland, Busscher \textit{et al.} (2006) identified among 70 staphylococci of horses the species \textit{Staphylococcus lentus}, \textit{S. capitis}, \textit{S. kloosii}, \textit{S. cohnii} subsp. \textit{cohnii}, \textit{S. warneri} or \textit{S. haemolyticus} which were resistant to methicillin. In Japan (without above-mentioned species), MR \textit{S. saprophyticus} and \textit{S. xylosus} were detected in 44 horses of 8 riding clubs (Yasuda \textit{et al.}, 2000). In general, some bacteria have been shown to inhibit the growth of the other bacteria due to the production of a variety of inhibitory substances like bacteriocins, bacteriolytic enzymes and low molecular weight antibiotics (Zaria, 1993). Bacteriocins, produced by enterococci mostly termed enterocins, were previously studied; however their detail studies have been developed during recent 15 years (Franz \textit{et al.}, 2007). \textit{In vitro} as well as \textit{in vivo} effect of enterocin-producing strains and their enterocins

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was previously reported by Aymerich et al. (1996), Audisio et al. (2000), Cintas et al., (2000), Lauková et al. (1993), Herich et al. (2010). Moreover, the antibacterial, anthelmintic effect of plant extracts, especially oregano and sage, has been already published (McGaw et al. 2007; Marcin et al. 2006). Our study was aimed at finding whether fecal strains of MR Staphylococcus xylosus from horses can be sensitive to enterocins and herbal substances (oregano, sage) to bring information about this lactic acid producing species from horse’s niche in the framework of the basic research.

MATERIALS AND METHODS

Sampling and microbial procedures

Staphylococci were isolated from faecal samples of 122 horses (farms mostly in Western Slovakia). Sampling and animal handling followed the Guide for the Care of Animals accepted by Ethic Commission (Institute of Animal Physiology Slovak Academy of Sciences, Košice, Slovakia) and by the State Veterinary and Food Institute of Slovak Republic. The samples were treated using the standard microbiological method according to ISO 6888. Serial ten-fold dilutions (in 0.85 % saline solution) were plated onto Mannitol Salt agar (MSA, Becton and Dickinson, USA) and cultured at 37°C for 48 h. Selected colonies were genotyped by PCR using Techgene KRD thermocycler (Techne, UK). DNA from each strain was used as a template for PCR analysis according to the protocol provided by Aymerich et al. (2003). For PCR-amplification of the species, the sequences of the primer pairs were Sxyl F3 5’-AAGTCGGTTGAAAAACCTAAA-3’, Sxyl R2 5’-CATTGACATATTGTATTCAT-3’; the product size of 217 bp. Positive control was S. xylosus SX SO3/1/1M/2 (our isolate, Lauková et al., 2010).

Lactic acid production was tested by spectrophotometric method according to Pryce (1969) and expressed in mmol/l.

Sensitivity to enterocins and herbal substances

The producer strains of enterocins as well as their characterization are shown in Table 1. Briefly, partially purified enterocins (Ents) were prepared by the following procedures. A 16-18 h culture (300 ml) of E. faecium EK 13-CCM 7419 (Mareková et al., 2003), EF 9296 (Marcináková 2006), EF 55 (Strompfová and Lauková, 2007), CCM 4231 (Lauková et al., 1993), AL 41 (Mareková et al., 2007) strains in MRS or Todd-Hewitt broth (Merck, Germany; Becton and Dickinson, USA) were centrifuged for 30 min at 10 000 x g in order to remove the cells. After adjusting of the supernatant to pH 5.0 (5.5 for AL 41), ammonium sulphate was gently added to the supernatant (40 % - w/v saturation). The mixture was stirred at 4°C for 2 h (EK 13, EF 9296), for 24 h (EF 55, CCM 4231), at 21°C for 1 h (Ent AL 41 =Ent M). After centrifugation at 10 000 x g for 30 min, the resulting pellet was resuspended in minimal volume of sodium phosphate buffer (pH 6.5). The inhibitory activity of Ents against S. xylosus isolates (sensitivity of strains to Ents) was performed by the agar spot test according to De Vuyst et al. (1996) and quantified. The antimicrobial titre of Ents was defined as the reciprocal of the highest twofold dilution producing a distinct inhibition of the inhibitory lawn and was expressed in arbitrary units per millilitre of culture medium (AU/ml). E. avium EA5 (our isolate from piglet) was used as a

Table 1: Enterocin-producing strains used in this study

<table>
<thead>
<tr>
<th>Enterocin</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ent M</td>
<td>Enterococcus faecium AL41</td>
<td>Mareková et al. (2007)</td>
</tr>
<tr>
<td>Ent A (P)</td>
<td>E. faecium EK13 (CCM7419)</td>
<td>Mareková et al. (2003)</td>
</tr>
<tr>
<td>Ent 4231</td>
<td>E. faecium CCM 4231</td>
<td>Lauková et al. (1993)</td>
</tr>
<tr>
<td>Ent EF55</td>
<td>E. faecium EF55</td>
<td>Strompfová a Lauková (2007)</td>
</tr>
<tr>
<td>Ent 9296</td>
<td>E. faecium EF 9296</td>
<td>Mareková et al. (2003)</td>
</tr>
</tbody>
</table>
bacteriocin-sensitive indicator strain (at the amount of 200 μl of an 18 hour culture of each indicator strain) to determine bacteriocin activity levels; the bacteriocin activity of tested \( \text{Ents} \) varied between 1600 - 25600 AU/ml (activity for individual enterocins is specified in Table 2).

Sensitivity of the strains to oregano and sage substances (10 μl of both) was also tested by the agar spot method (De Vuyst et al., 1996) using Brian Heart Infusion agar (1.5 and 0.7 %; Becton and Dickinson); but an inhibitory effect of essentials was expressed as an average value of an inhibitory zone in mm. \textit{Salvia officinalis} (24 % thujone, 18 % borneol, 15 % cineol) and \textit{Origanum vulgare} (55 % carvacrol, both from Calendula a.s., Nová Ľubovňa, Slovakia) were kindly provided by Dr. Šalamon and Dr. Poráčová (University of Prešov, Slovakia).

### Table 2: Methicillin-resistant \textit{Staphylococcus xylosus}, isolates from horses, their sensitivity to enterocins, herbal substances and lactic acid production (in mmol/l)

<table>
<thead>
<tr>
<th>Strains</th>
<th>\text{A(P)}</th>
<th>\text{55}</th>
<th>\text{M}</th>
<th>\text{9296}</th>
<th>Oregano</th>
<th>Sage</th>
<th>LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SX2A/2</td>
<td>100</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (33)</td>
<td>+ (13)</td>
<td>1.22±0.005</td>
</tr>
<tr>
<td>SX4A/3</td>
<td>3200</td>
<td>400</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>+ (15)</td>
<td>1.16±0.004</td>
</tr>
<tr>
<td>SX4A/5</td>
<td>100</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (31)</td>
<td>+ (14)</td>
<td>1.16±0.004</td>
</tr>
<tr>
<td>SX5A/1</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (30)</td>
<td>+ (12)</td>
<td>1.22±0.002</td>
</tr>
<tr>
<td>SX5A/2</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (30)</td>
<td>+ (13)</td>
<td>1.16±0.003</td>
</tr>
<tr>
<td>SX6A/1</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (25)</td>
<td>+ (10)</td>
<td>1.18±0.004</td>
</tr>
<tr>
<td>SX7A/3</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (29)</td>
<td>+ (9)</td>
<td>1.02±0.003</td>
</tr>
<tr>
<td>SX22A/1</td>
<td>100</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (30)</td>
<td>+ (12)</td>
<td>1.21±0.003</td>
</tr>
<tr>
<td>SX44A/1</td>
<td>ni</td>
<td>100</td>
<td>400</td>
<td>100</td>
<td>1600</td>
<td>+ (30)</td>
<td>0.92±0.003</td>
</tr>
<tr>
<td>SX56A/7</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (25)</td>
<td>+ (8)</td>
<td>1.09±0.002</td>
</tr>
<tr>
<td>SX56A/3</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>+ (17)</td>
<td>+ (12)</td>
<td>1.13±0.003</td>
</tr>
<tr>
<td>SX56A/4</td>
<td>ni</td>
<td>800</td>
<td>1600</td>
<td>ni</td>
<td>12 800</td>
<td>+ (26)</td>
<td>+ (11)</td>
</tr>
</tbody>
</table>


| ni - no inhibition; +: the strain was sensitive to herbal substances; (): size of inhibitory zone in mm. Partially purified enterocin (\text{Ent}) \text{M} produced by \textit{Enterococcus faecium} AL 41, \text{Ent} \text{A(P)} produced by \textit{E. faecium} EK13 (CCM 7419), \text{Ent} CCM 4231 (\textit{E. faecium} CCM 4231), \text{Ent} 55 (\textit{E. faecium} EF 55), \text{Ent} 9296 (\textit{E. faecium} 9296). The activity of \text{Ents} against the main indicator strain was from 1 600 up 25 600 AU/ml.

RESULTS AND DISCUSSION

The counts of staphylococci in fecal samples of 122 horses reached \( \log_{10} 2.03±0.42 \) cfu/g. Twelve staphylococcal isolates were found to be MR and they were taxonomically allotted to the species \textit{S. xylosus}. This species belongs to coagulase-negative staphylococci (CoNS). There is limited information concerning the occurrence of staphylococci in horse digestive system; e.g. Vengust et al. (2006) detected 126 MR CoNS from 300 horses. The occurrence of \textit{S. xylosus} was also detected in horses in Japan (Yasuda et al., 2000). The isolated strains produced lactic acid (LA) in the range of 0.92 - 1.22 mmol/l with the average value of 1.04±0.004 mmol/l meaning that the LA production was properly balanced. Staphylococci are lactic acid producing bacteria, but usually they produce less LA than lactobacilli or enterococci (Lauková and Kuncová, 1991). Here, the high level of LA production was noted; even higher LA values than by ruminal staphylococci were noted (Lauková and Kmeť, 1992). It is assumed that also among staphylococci, the quantity of LA production can be strain- or species-dependent. Six strains (Table 2) were not sensitive to \text{Ents} used; however, they were sensitive to oregano and sage substances reaching inhibitory zones in size of 17-31 mm (Table 2). The other strains were sensitive at least to 3 of 5 \text{Ents} tested with the inhibitory activity of 100 - 12 800 AU/ml, and they were also sensitive to both herbal substances (Table 2). The most sensitive strains were SX4A/3, SX44A/1 and SX56A/4; they were sensitive to 4-5 \text{Ents}. The growth of \textit{S. xylosus} SX56A/4 was inhibited by \text{Ent} \text{A(P)}, \text{Ent} 55 and \text{Ent} 9296 with
activities of 800, 1600 and 12,800 AU/ml; the later value was the highest activity reached during our testing. The strain SX4A/3 was inhibited by all 5 EntS with activities of 100, 200, 400 and 3 200 AU/ml. However, the sizes of its inhibitory zones concerning the herbal substances were the lowest comparing with other strains (Table 2). The strains of S. xylosus were sensitive to both oregano and sage substances, but larger inhibitory zones were measured at oregano compared to sage (Table 2). The size of inhibitory zones due to oregano was in the range of 17 - 38 mm (28 mm at average) and due to sage in the range of 5-14 (12 mm at average). S. xylosus strains were less sensitive to EntS than to herbal substances. In spite of the fact, that in our study the presence of Mec gene was not checked yet, the strains were phenotypically MR. Slobodniková et al. (2001) reported a higher occurrence of MR staphylococci in the group of CoNs. Nascimento et al. (2006) reported lower frequency of MR CoNs inhibited by bacteriocins-staphylococci when compared to MR S. aureus strains. They found Pep 5 as the most effective bacteriocin against MR CoNs from clinical sources. They also summarized that staphylococci from different origins showed variable sensitivity to the bacteriocins. Bacteriocin resistance is a complex phenotype involving alterations in cell wall and/or cytoplasmic membrane (Crandall and Montville, 1998). Finland et al. (2002) has recently described that the presence of bacteriocin genome of extra immunity genes expressed without a cognate bacteriocin expands the bacteriocin resistance of the strain possessing these genes. Oregano and sage are good inhibitors of not only Gram-positive but also Gram-negative species; it was confirmed in our previous studies under both in vitro and in vivo conditions (Szabóová et al., 2008; Pogány Simonová et al., 2010).

CONCLUSION

It is indicated that oregano as well as sage, given at the concentrations tested, are able to inhibit the growth of MR S. xylosus. In spite of not a huge sensitivity of the strains to EntS tested here MR S. xylosus were sensitive at least to 3 EntS used. Although a limited number of strains were examined in this study and not all known EntS were available, nevertheless our results expand recent knowledge and suggest continuing in this research.

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