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METHICILIN-RESISTANT *STAPHYLOCOCCUS XYLOSUS* ISOLATED FROM HORSES AND THEIR SENSITIVITY TO ENTEROCINS AND HERBAL SUBSTANCES

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ABSTRACT

Methicillin-resistant (MR) strains *S. xylosus* from faeces of horses were tested for their sensitivity to enterocins (*Ents*) and herbal substances (oregano, sage) to contribute to basic research related to *Ents* and horses microbiota. The counts of staphylococci in fecal 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 *S. xylosus*. The strains produced lactic acid at average of 1.04±0.004 mmo/l. Six strains were not sensitive to *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 *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 *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 *Ents* than to herbals.

Key words: horses; *Staphylococcus xylosus*; enterocins; oregano; sage; sensitivity

INTRODUCTION

In general, microbiology of the equine gastrointestinal tract is poorly characterized. Garret *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 10^6cfu/g of Gram-negative rods. Staphylococci belong to the phylum Firmicutes, to the genus Staphylococcus and to the Family Staphylococcacae (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 The counts of staphylococci in faecal samples from farm horses reached about log10 3.0 cfu/g (unpublished results).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, methicilin-resistant (MR) staphylococci belong to one of the most serious therapeutical 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 *et al.* (2006) identified among 70 staphylococci of horses the species *Staphylococcus lentus, S. capitis, S. kloosii, S. cohnii* subsp. *cohnii, S. warneri* or *S. haemolyticus* which were resistant to methicilin. In Japan (without above-mentioned species), MR S. *saprophyticus* and *S. xylosus* were detected in 44 horses of 8 riding clubs (Yasuda *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 *et al.,* 2007). *In vitro* as well as *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, antihelmintic 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, Cockeysville, USA) and cultured at 37ºC 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`-AAGTCGGTTGAAAACCTAAA-3´, Sxyl R2 5´-CATTGACATATTGTATTCAT-3´; the product size of 217 bp. Positive control was *S. xylosus* SX S03/1/1M/2 (Lauková *et al.,* 2010). The PCR products (10 µl each) were separated by electrophoresis in 0.8 % agarose gels (Sigma, Germany) buffered with 1xTAE (Merck, Germany) containing 1 µg/ml ethidium bromide (Sigma). The molecular mass standard (Promega, USA) was used according to the manufacturers' instruction.

Testing of methicilin sensitivity/resistance, lactic acid production

Resistance/sensitivity to methicilin was tested by the agar disk-diffusion method (NCCLS 2006) using Brian Heart Infusion agar enriched with 10 % of defibrinated sheep blood. Antibiotic disks used were supplied by Fluka-Biochemika (Mec, 10 µg). After incubation at 37ºC for 18 h, the strains were classified as resistant or sensitive (according to the manufacturers´ instruction). 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 (Marciňáková 2006), EF 55 (Strompfová and Lauková, 2007), CCM 4231 (Lauková *et al*., 1993), AL 41 (Mareková *et al*., 2007) strains in MRS or Todd-Hewit 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

Enterocin	Strain	Source
Ent M	*Enterococcus faecium* AL41	Mareková *et al*. (2007)
Ent A (P)	*E. faecium* EK13 (CCM7419)	Mareková *et al*. (2003)
Ent 4231	*E. faecium* CCM 4231	Lauková *et al.* (1993)
Ent EF55	*E. faecium* EF55	Strompfová a Lauková (2007)
Ent 9296	*E. faecium* EF 9296	Mareková *et al.* (2003)

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 *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. *Salvia officinalis* (24 % thujone, 18 % borneol, 15 % cineol) and *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: Methicilin-resistant *Staphylococcus xylosus*, isolates from horses, their sensitivity to enterocins, herbal substances and lactic acid production (in mmol/l)

Strains	4231	A(P)	55	M	9296	Oregano	Sage	LA
SX2A/2	100[a]	ni	ni	ni	ni	+ (33)	+ (13)	1.22±0.005
SX4A/3	3200	400	100	100	200	+(15)	+ (5)	1.16±0.004
SX4A/5	100	ni	ni	ni	ni	+(31)	+ (14)	1.16±0.004
SX5A/1	ni	ni	ni	ni	ni	+(30)	+ (12)	1.22±0.002
SX5A/2	ni	ni	ni	ni	ni	+(30)	+ (13)	1.16±0.003
SX6A/1	ni	ni	ni	ni	ni	+(25)	+ (10)	1.18±0.004
SX7A/5	ni	ni	ni	ni	ni	+(29)	+ (9)	1.02±0.003
SX22A/1	100	ni	ni	ni	ni	+(30)	+ (12)	1.21±0.003
SX44A/1	ni	100	400	100	1600	+(30)	+ (12)	0.92±0.003
SX56A/7	ni	ni	ni	ni	ni	+(25)	+ (8)	1.09±0.002
SX56A/3	ni	ni	ni	ni	ni	+(17)	+ (12)	1.13±0.003
SX56A/4	ni	800	1 600	ni	12 800	+(26)	+(11)	1.21±0.009

ni - no inhibition ; +: the strain was sensitive to herbal substances; (): size of inhibitory zone in mm. Partially purified enterocin (*Ent*) M produced by *Enterococcus faecium* AL 41, *Ent* A (P) produced by *E. faecium* EK13 (CCM 7419), *Ent* CCM 4231 (*E. faecium* CCM 4231), *Ent* 55 (*E. faecium* EF 55), *Ent* 9296 (*E. faecium* 9296). The activity of *Ents* against the main indicator strain was from 1 600 up 25 600 AU/ml. [a]bacteriocin activity in Arbitrary units per ml. *Salvia officinalis* (24 % thujone, 18 % borneol, 15 % cineol); *Origanum vulgare* substances (55 % carvacrol, both from Calendula a.s., Nová Ľubovňa, Slovakia) kindly provided by Dr. Šalamon, Dr. Poráčová (University of Prešov, Slovakia)

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 *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 *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 mmo/l with the average value of 1.04±0.004 mmo/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 *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 *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 *Ents*. The growth of *S. xylosus* SX56A/4 was inhibited by *Ent* A(P), *Ent* 55 and *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. Slobodníková *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-staphylococcins 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). Fimland *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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