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Short Communication

Essential Oils in the Control of Infections by *Staphylococcus xylosus* in Horses

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ABSTRACT

The antimicrobial activity of nine essential oils (cinnamon, palmarosa, Indonesian and Madagascar cloves, niaouli, peppermint, oregano, rosemary, and sauce thyme) against *Staphylococcus xylosus* isolates obtained from the nasal mucosa of healthy and diseased horses has been valued. A total of 27 isolates with different resistance profile (susceptible, resistant to one antimicrobial class, resistant to ≥2 antimicrobial classes) were analyzed. An initial screening study was carried out to determine the antimicrobial activity of essential oils against 10 *S. xylosus* isolates, following the disk-diffusion method. Five essential oils with major inhibitory zone were selected, and the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against the 27 isolates were performed by a broth microdilution method. All the essential oils, except rosemary (inhibitory zone diameter of 6 mm for 100% of isolates), showed antimicrobial activity against *S. xylosus*. The MIC and MBC values were obtained for cinnamon, clove, peppermint, oregano, and sauce thyme. Our results show that sauce thyme (0.07 ± 0.05% and 0.31 ± 0.39%, respectively) and oregano (0.12 ± 0.04% and 0.52 ± 0.42%, respectively) presented the lowest MIC and MBC average values. No significant differences were observed between the inhibitory activity and the resistance profile of the isolates. The sauce thyme and oregano could be used in the control of *S. xylosus* infection in horses. More studies are necessary to establish their clinical potential use, as well as their dose and application options.

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1. Introduction

Coagulase-negative staphylococci (CoNS) are opportunist pathogens commonly found in the nasal mucosa and skin of healthy horses. These bacteria are frequently exposed to different antibiotics commonly used in animals. This exposure can be responsible for the acquisition of antimicrobial resistance among commensal flora of healthy animals [1]. Carrier horses could play a fundamental role in the transmission of resistant CoNS to other animals and their careers, with special attention to the methicillin-resistant isolates [2,3].

Clinical importance of CoNS in veterinary medicine is not completely clarified. Cases of skin abscess, mastitis, wound, respiratory and uterine infections caused by methicillin-susceptible and methicillin-resistant strains have been described [3]. It is shown that animal nasal colonization is a risk factor for clinical infection development [2], and *Staphylococcus xylosus, Staphylococcus epidermidis, Staphylococcus sciuri*, and *Staphylococcus vitulinus* are some of the species most frequently isolated from healthy and diseased horses [1,3].

The results of this work have been presented in the International Conference on Antimicrobial Research (ICAR) 2014, Madrid (Spain).

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Essential oils (EOs) are currently used as food additives, although they have been traditionally used to control gastrointestinal and respiratory infections given their intestinal availability and their volatile nature [4]. Essential oils have showed a notable activity against diverse *Staphylococcus* species involved in food-borne diseases and mastitis in cows [5,6]. However, any standardized method to study the antimicrobial activity of EOs *in vitro* exists. Following antibiotic guidelines, most authors use the disk-diffusion method as a screening test and microdilution method as a quantitative test [5,6]. However, a disagreement about the concordance of both of the methods has been obtained, being necessary a regression study using 50 to 500 isolates to compare them [7].

In this work, an *in vitro* study was carried out to evaluate the antimicrobial activity by the disk-diffusion method of nine EOs against *S. xylosus* species isolated from horses. Furthermore, the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC), following the broth microdilution method, were determined for those EOs with the best results in the aforementioned assay.

2. Materials and Methods

2.1. Bacterial Isolates

A total of 27 *S. xylosus* isolates obtained from the nasal mucosa of healthy and diseased horses, assisted at a Veterinary Clinical Hospital, were studied. Seven out of the 27 isolates were susceptible to different antimicrobials commonly used in equine medicine; fifteen out of them were resistant to one antimicrobial class (86% β-lactams), and five were resistant to two or more antimicrobial classes.

2.2. Essential Oils

Nine EOs extracted from plants and spices of natural origin (Marnys S.A., Cartagena, Spain), with a purity ≥98%, were tested: cinnamon (*Cinnamomum zeylanicum*, 96.7% cinnamaldehyde), palmarosa (*Cymbopogon martinii*, 82.9% geraniol), Indonesian and Madagascar cloves (*Eugenia caryophyllata*, 75.3% eugenol), niaouli (*Melaleuca viridiflora,* 64.4% α-terpinolene), peppermint (*Mentha piperita,* 50% menthol), oregano (*Origanum vulgare,* 28.5% thymol and 19.5% carvacrol), rosemary (*Rosmarinus officinalis,* up to 50% of 1.8 cineol), and sauce thyme (*Thymus zigys,* 48% thymol and 25.5% p-cymene).

2.3. Disk-Diffusion Method

An initial screening study was carried out to determine the antimicrobial activity of nine EOs against 10 out of the total of *S. xylosus* isolates (n = 27). The Clinical and Laboratory Standards Institute guidelines [8] for antibiotic tests were followed with brief modifications: each product was tested individually in 5 cm of diameter Mueller-Hinton agar plates (Oxoid Ltd, Hampshire, England) to avoid interactions between volatile oil's compounds. Plates were inoculated with 20 μL of a 1.5×10^8 CFU/mL bacterial suspension. A sterile blank disk of 6 mm in diameter (Oxoid Ltd), previously impregnated with 15 μL of pure EO (EO disk), was placed on the plate [9]. Once sealed, all plates were incubated in aerobiosis at 35°C for 24 hours, and the inhibitory zone diameter was determined.

The bacterial concentration was confirmed by plate count method. All the assays included a positive growth control (plate with the bacteria without EO disk) and a negative control (plate with EO disk, without bacteria). The reference strains of *S. aureus* ATCC 25923 and penicillin disk 10 IU (Oxoid Ltd) were also used as controls.

2.4. Broth Microdilution Method

Based on the Clinical and Laboratory Standards Institute protocol [8], serial double dilutions of each EO were prepared (total volume of 10 mL) in Mueller-Hinton broth (Oxoid Ltd) to achieve a final concentration between 16% and 0.03% (v/v). Plates of 96 wells (Greiner Bio-one International, Austria) were used; in each well, 100 μL of the EO was mixed with 100 μL of bacterial suspension (10^8 CFU/mL). The final bacterial concentration was 5×10^5 CFU/mL. In all the assays, a positive control (containing bacteria but not EO), a negative control (containing EO without bacteria), and a quality control (*S. aureus* ATCC 25923 and penicillin G sodium salt [Sigma–Aldrich Co, LLC., St. Louis] were included. The final bacteria concentration was confirmed by the plate count method.

After incubation at 35°C for 24 hours, the MIC was determined as the lowest oil concentration able to inhibit the visible bacterial growth in the wells. To determine the MBC, 10 μL from the latter four wells without visible bacterial growth was inoculated in Muller-Hinton agar. It was incubated at 35°C for 24 hours, and the MBC was calculated as the lowest oil concentration able to destroy the 99.9% of the inoculum in the well, based on the total absence of colonies in agar plate.

2.5. Statistical Analysis

All the assays were carried out in triplicate, and the results were expressed as average of the inhibitory zone ± standard deviation. Statistical analyses were performed using SPSS 15.0 software for Windows (IBM Company, New York), and significance was set at $P < .05$. An analysis of variance (ANOVA) test was used to compare the averages of the inhibitory zone of each oil for *S. xylosus* ($P < .05$). The MIC_{50}, the MBC_{50}, the mode, and the range (minimum value–maximum value) of the selected EO against *S. xylosus* were calculated. The MIC_{90} and the MBC_{90} could not be determined in any case. Finally, ANOVA and Tukey tests were used to determine the possible relationship among the EO activity and the resistance profile of the isolates (susceptible, resistant to one antimicrobial class, and resistant to ≥2 antimicrobial classes).

3. Results

Table 1 shows the average inhibitory zone obtained for the nine EOs by the diffusion disk–plate method, grouped in three categories, following the Moreira et al.

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