Bulgarian Journal of Veterinary Medicine, 2023, **26**, No 1, 115−121
ISSN 1311-1477; DOI: 10.15547/bjvm.2020-0144

Short communication

A NEW SOLUTION FOR CONTROL OF RESPIRATORY INFECTION BY *STREPTOCOCCUS EQUI* ISOLATED FROM HORSES IN NORTHERN IRAN

M. YAGHOOBPOUR[1], L. FOZOUNI[1] & P. GHAEMI[2]

[1]Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran;
[2]Department of Laboratory Sciences, Gorgan Branch, Islamic Azad University, Gorgan, Iran

Summary

Yaghoobpour, M., L. Fozouni & P. Ghaemi, 2023. A new solution for control of respiratory infection by *Streptococcus equi* isolated from horses in northern Iran. *Bulg. J. Vet. Med.,* **26**, No 1, 115−121.

Respiratory infections caused by *Streptococcus equi* are one of the most important diseases in the equine industry. The increasing rate of antibiotic resistance among this bacterial species necessitates new antimicrobial agents. In this research, nasopharyngeal samples were taken from horses (n=90) in some areas of the Golestan Province, Iran. After isolation and identification of *S. equi* strains, antibiotic resistance of the isolates was evaluated using the Kirby-Bauer method. The antibacterial effect of lavender essential oil was investigated by broth microdilution assay. The frequency of streptococcal strains was 27.8%. The most frequent isolates were *S. equi subsp. equi* (72%) and *S. equi subsp. zooepidemicus* (28%). The frequency of *S. equi* was highest in horses aged less than 2 years (69.3%) and thoroughbred Turkmen horses (61.5%). The highest rate of resistance was observed against amoxicillin, while the highest rate of sensitivity was to ceftriaxone. Furthermore, the essential oil of lavender inhibited 90% of the isolates at concentrations ≥2048 µL/mL. Our study revealed that the frequency of antibiotic-resistant *S. equi* isolates was relatively high in the studied population of horses in Iran. It was concluded that essential oil of lavender possessed more potent *in vitro* activity than the common antibiotics for treatment of infections caused by *S. equi* subspecies.

Key words: lavender, respiratory infection, *Streptococcus equi*, traditional medicine

Today, the horse industry is considered a purely economic profession in Iran, particularly in the Golestan Province, which is major habitat of Turkmen horses. Therefore, horses can influence the economy of the residents both directly and indirectly, which signifies the importance of policymaking to determine prevalence of diseases in these animals. One of the most important diseases in the livestock industry is infections caused by *Streptococcus equi*, such as strangles (Mohammadi *et al*., 2016). The disease is highly contagious and widespread worldwide. Horses can be infected at any age, but young horses are more susceptible (Ijaz *et al*., 2012; Fang *et al*., 2019). The most important feature of this disease is inflammation of the upper respiratory tract; the bacterium multiplies in the respiratory mucosa, affects the lymph nodes in the area and produces purulent abscesses along the lymph nodes. After a few days, the symptoms of lymphatic system involvement appear in form of enlarged submandibular and retropharyngeal lymph nodes. There are also extensive traces of purulent abscesses in the liver, lungs, peritoneum and pleura (Ma *et al*., 2017). *S. equi subsp. zooepidemicus* is an opportunistic commensal and pathogen of the upper respiratory tract in horses, particularly during stress or immunosuppresion, and it can also be transmitted to humans. These Lancefield group C streptococci are beta-haemolytic (Yi *et al*., 2016; Remmington & Turner, 2018). Undoubtedly, horse breeding without considering proper management and health aspects is illogical (Ijaz *et al*., 2012; Commons *et al*., 2014; Mohammadi *et al*., 2016). Due to the widespread damages caused by these infections, the use of antibiotics is mandatory. On the other hand, the increasing rate of antibiotic resistance promotes the need for discovery of novel antibacterials, such as medicinal herbs (Waller., 2014). Therefore, the use of different antimicrobial compounds like medicinal herbs to prevent or control such resistance is recommended.

Iran is a country rich in medicinal plants, including lavender, which is mentioned in the Canon of Medicine by Avicenna. Lavender is native to the Mediterranean region but can be also found in Africa and India (Hamedi *et al*., 2013; Donelli *et al*., 2019). Early studies have shown that the aerial parts of lavender have stronger antimicrobial effects than other parts of the plant. Additionally, the leaves of this plant contain large amounts of aromatic alcohols, flavonoids and organic acids such as carnosic acid and saponins, which have proven antibacterial properties (Ozcan, 2003; Stierlin *et al*., 2020).

Considering the increasing prevalence of infections caused by *S. equi* in horses in some areas of the Golestan Province this study was a cross-sectional survey to evaluate the antibacterial properties of lavender essential oil on these strains isolated from horses.

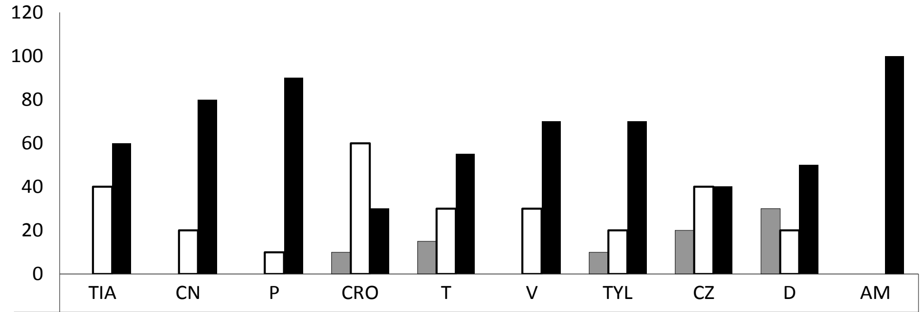
Ninety samples were obtained from horses with or without respiratory symptoms in some animal farms of the Golestan Province, Iran, during a six-month period in 2018–2019. Specimens were taken using sterile nasopharyngeal swabs (after cleaning the anterior area with disinfectant). The swabs were placed in brain heart infusion broth (BHI, Merck, Germany) containing 10 mg/L gentamicin and 15 mg/L nalidixic acid (Sigma-Aldrich, USA), and later transferred to the laboratory. After four hours of incubation at 37 ^{o}C and in anaerobic conditions, the samples were cultured in blood agar (Merck, Germany) containing 5% sheep's blood for 24 hours at 37 ^{o}C and 5% CO_2. Then, *S. equi* strains were identified based on the type of haemolysis on blood agar and results of Gram staining, catalase test, sensitivity to bacitracin/SXT, hippurate hydrolysis, growth in 6.5% sodium chloride and fermentation of salicin, sucrose, lactose, sorbitol, trehalose and starch.

Antibiotic susceptibility was evaluated by the agar disk diffusion (Kirby-Bauer) method. First, bacterial suspension equivalent to 0.5 McFarland standard was cultured on Mueller Hinton agar containing 5% blood three times while rotating the plate 60 degrees after each inoculation. The following antibiotic disks were purchased from Padtan Teb Co. (Iran): amoxicillin (AM, 25 µg), vancomycin (V, 30 µg), penicillin (P, 375 mg), cephalexin (CN, 30 µg), doxycycline (D, 30 µg), tetracycline (T, 30 µg), ceftriaxone (CRO, 30 µg), cefazolin (CZ, 30 µg) tiamulin (TIA, 30 µg) and tylosin (TYL, 25 µg). After 24 hours of incubation at 37 ^{o}C and 5% CO_2, diameter of growth inhibition zone around the disks was measured and results were analysed according to the Clinical and Laboratory Standards Institute M100-S22 guidelines (CLSI, 2015). In all experiments, the *Streptococcus pneumoniae* ATCC49619 strain was used as control.

In this research, lavender leaves were collected in a sunny day from fields of Gilan Province, Iran. After separating leaves, they were washed, dried and finally ground. For extraction of essential oil, 25 g of powdered plant components were mixed with 250 mL of sterile distilled water for three consecutive days. The essential oil of lavender (50 g) was obtained by distillation using a Clevenger-type apparatus (three times), recovered and dehydrated with sodium sulfate and stored in a dark glass container at 4 ^{o}C until analysis. The mixture was filtered using Whatman No. 2 (USA) filter papers. The obtained essential oil was weighed and then stored at 4 ^{o}C. The antibacterial effect of lavender essential oil was evaluated using the broth microdilution method (CLSI, 2015). For this purpose, various dilutions ranging from 8 to 4096 µl/mL were prepared from the lavender essential oil. Next, 50 µL of successive dilutions of the essential oil were transferred into wells of a 96-well microplate containing 50 µL Mueller Hinton broth. Then, 50 µL of *S. equi* bacterial suspension with a turbidity equivalent to 0.5 McFarland standard were inoculated into the wells. After incubation at 37 ^{o}C and in anaerobic conditions, the inhibitory effect of lavender essential oil was assessed by reading absorbance at 630 nm using a Plate Reader (BioTec, Germany). The minimum concentration which inhibited bacterial growth up to 90% in comparison with the positive control was considered as $MIC_{90.}$ The negative control well contained essential oil with Mueller Hinton broth, and the positive control well contained Mueller Hinton broth with bacterial suspension.

Of 90 specimens collected from horses, 25 (27.8%) *S. equi* strains were isolated based on the microbiological testing. The frequency of *S. equi* isolates was highest among horses aged <2 years (69.3%) and thoroughbred Turkmen horses (61.5%). The most common isolates among horses were *S. equi subsp. equi* (72%) and *S. equi subsp. zooepidemicus* (28%). As shown on Fig. 1, the isolates were mostly resistant to amoxicillin and penicillin, while they were highly sensitive to ceftriaxone. There was no significant difference in terms of resistance to ceftriaxone between bacterial subspecies (P=0.036).

The results showed that the lavender essential oil could inhibit 90% of *S. equi* isolates at concentrations ≥ 2048 (MIC_{90}: 2048 µL/mL). As shown in Table 1, the mean MIC of lavender essential oil against the isolates was 1038 µL/mL so that the highest growth changes were observed at concentrations of 256 µL/mL and 512 µL/mL. Generally, in the microdilution test, lavender essential oil ex-



	TIA	CN	P	CRO	T	V	TYL	CZ	D	AM
I	0	0	0	10	15	0	10	20	30	0
S	40	20	10	60	30	30	20	40	20	0
R	60	80	90	30	55	70	70	40	50	100

Fig. 1. Relative frequency (%) of antibiotic resistance among *S. equi* isolates. I: intermediate, R: resistant, S: sensitive; TIA: tiamulin, CN: cephalexin, P: penicillin, CRO: ceftriaxone, T: tetracycline, V: vancomycin, TYL: tylosin, CZ: cefazolin, D: doxycycline, AM: amoxicillin.

Table 1. Inhibitory effects of the lavender essential oil against 25 *S. equi* subsp. isolates (3×10^8 CFU/mL)

Essential oil (µL/mL)	No growth	Growth
	Number (%)	Number (%)
256	4 (16%)	21 (84%)
512	6 (24%)	19 (76%)
≥2048	23 (92%)	2 (15%)

hibited significant inhibitory effects on drug-resistant *S. equi* subspecies (P<0.01).

In Iran, researchers reported the first outbreak of strangles among horse stalls in Tehran, with an estimated prevalence of 80 to 100% (Noormohamadzadeh *et al.*, 1992). In this study, *S. equi* was isolated from 27.8% of specimens. The frequency of *S. equi subsp. equi* was almost 2.5-fold higher than that of *S. equi subsp. zooepidemicus*. It seems that horses were contaminated from infected owners as previous research had confirmed the zoonotic transmission of the bacterium (Skive *et al.*, 2017). In a study in Bosnia and Herzegovina, 118 of 1852 horses had strangles; nine of whom had mesenteric lymphadenitis (Vukovic *et al.*, 1961). In a study on 179 horses between 1985 and 1988, at least 27.4% of the herds were dealing with strangles outbreak (Jorm *et al.*, 1990).

A study in Japan reported *S. equi* as the cause of subcutaneous lymphadenitis-related suffocation of horses (Anzai *et al.*, 1997). In 2012, a study on horses demonstrated that *S. equi* was significantly more prevalent in specimens from mandibular lymph nodes than in those from nasal secretions (Ijaz *et al.*, 2012).

Antibiotic therapy has been used against such infections as well as to prevent the transmission of infection from animal to humans (Hassan, 2014); however, as shown in our study, the increasing rate of antibiotic resistance among *S. equi* signifies the need for new antimicrobial agents without notable side effects.

Medicinal plants such as lavender have long been used as natural antimicrobial compounds (Rodriguez *et al*., 2005; Oorojalian *et al*., 2009). A study claimed that the methanolic extract of flowering branches of lavender from polluted areas of Iran had no antimicrobial activity (Farahzadi *et al*., 2014). However, lavender has long been used in traditional Iranian medicine for treating some neurological diseases such as epilepsy, insomnia and some infections (Hamedi *et al*., 2013). Our findings indicate that the lavender essential oil could inhibit growth of *S. equi* isolates in a dose-dependent manner so that more than 90% of the isolates were eliminated at concentrations of 2048 and 4096 μL/mL. Previous studies around the world have confirmed the antibacterial (Dorman *et al*., 1995; Ahmady-Abchin *et al*., 2012; Hossain *et al*., 2017) and antifungal (Adaszyńska-Skwirzyńska *et al*., 2018; Váczi *et al*., 2018) effects of lavender essential oil.

The findings of our study reveal that the frequency of antibiotic-resistant *S. equi* isolates in the studied population of horses in Iran was relatively high. This in turn may ultimately lead to economic recession and spread of infection. Therefore, monitoring prevalence rates is recommended in order to improve the quality of treatment of infections caused by the bacterium and to update epidemiological information. Given the antimicrobial effects of lavender essential oil against *S. equi*, further investigation on its efficiency for treatment of infections caused by this bacterium is suggested.

ACKNOWLEDGEMENTS

This study has been supported by the Research Council of the Islamic Azad University of Gorgan, Iran. We appreciate the cooperation of Gorgan's animal husbandries for the study.

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Paper received 23.09.2020; accepted for publication 29.12.2020

Correspondence:

Leila Fozouni,
Department of Biology,
Gorgan Branch,
Islamic Azad University,
Gorgan, Iran,
tel:+989111518674;
fax: +981133329496;
email: lili_kia@yahoo.com