SHORT COMMUNICATION

Detection of multidrug-resistant *Streptococcus equi* subsp. *zooepidemicus* in Cuba

Detección de *Streptococcus equi* subsp. *zooepidemicus* multirresistente a antimicrobianos en Cuba

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ABSTRACT: *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is an opportunistic pathogen capable of causing various infections in the respiratory, genital, and urinary tracts of horses and other animals. This species is considered responsible for many emerging zoonotic diseases. There has been an increasing circulation of multidrug-resistant *S. zooepidemicus* strains in horses, however, there is no information on *S. zooepidemicus* and its antimicrobial susceptibility profile in Cuban horses. Therefore, the objectives of this study were to report the isolation of *S. zooepidemicus* in a horse and to determine its antimicrobial susceptibility profile. A mare with pale mucous membranes, owned by a private producer in Melena del Sur, Mayabeque, was sampled, and a swab was taken from the genital tract. The isolate obtained was identified using the analytical profile index and mass spectrometry. The minimal inhibitory concentration (MIC) of 11 antibiotics (penicillin G, ampicillin, amoxicillin-clavulanate, cefquinome, imipenem, gentamicin, enrofloxacin, doxycycline, erythromycin, chloramphenicol, and vancomycin) was determined for the isolate. The isolate was identified as *S. zooepidemicus* and it was susceptible to all *β*-lactam, erythromycin, chloramphenicol, and vancomycin. The isolate presented a multidrug resistance profile to gentamicin, enrofloxacin, and doxycycline with MIC values of 16 µg/mL, 2 µg/mL, and 2 µg/mL, respectively. For the first time in Cuba, multidrug-resistant *S. zooepidemicus* was detected in the genital mucosa of a mare. The close interaction between humans and horses increases the risk of acquiring these multiresistant microorganisms or favoring their dissemination, thus this result should be considered in staff training.

Key words: *Streptococcus equi* subsp. *zooepidemicus*, antimicrobial resistance, mare.

RESUMEN: *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) es un patógeno oportunista capaz de causar diversas infecciones en los tractos respiratorio, genital y urinario en caballos y otros animales. Esta especie se considera responsable de muchas enfermedades zoonóticas emergentes. Recientemente, existe una creciente circulación de cepas de *S. zooepidemicus* multirresistentes a antimicrobianos en caballos, sin embargo, no existe información sobre *S. zooepidemicus* y su perfil de susceptibilidad a antimicrobianos, en caballos cubanos. Por tanto, los objetivos de este estudio fueron reportar el aislamiento de *S. zooepidemicus* en un caballo y determinar su perfil de susceptibilidad a antimicrobianos. Se muestreó una yegua perteneciente a un productor privado de Melena del Sur, Mayabeque, con mucosas pálidas y se tomó un hisopado del tracto genital. El aislado obtenido se identificó mediante el índice de perfil analítico y espectrometría de masas. Se determinó la concentración mínima inhibitoria (CMI) de 11 antibióticos (penicilina G, ampicilina, amoxicilina-clavulanato, cefquinoma, imipenem, gentamicina, enrofloxacina, doxiciclina, eritromicina, cloranfenicol y vancomicina) para el aislado. El aislado se identificó como *S. zooepidemicus* y fue sensible a todos los betalactámicos, eritromicina, cloranfenicol y vancomicina. Presentó un perfil de multirresistencia a gentamicina, enrofloxacina y doxiciclina con valores de CMI de 16 µg/mL, 2 µg/mL y 2 µg/mL, respectivamente. Por primera vez en Cuba se detecta *S. zooepidemicus* multirresistente a antimicrobianos en la mucosa genital de una yegua. La estrecha interacción entre humanos y caballos, aumenta el riesgo de adquirir estos microorganismos multirresistentes o favorecer su diseminación, por lo que este resultado debe ser considerado en la capacitación del personal con vínculo ocupacional.

Palabras clave: *Streptococcus equi* subsp. *zooepidemicus*, resistencia a antimicrobianos, yegua.

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Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) is a *β*-hemolytic *Streptococcus* belonging to the Lancefield group C (1). *S. zooepidemicus* is one of the most common pathogens infecting horses (2). It is also commonly isolated from bacterial infections in a wide range of animal species such as dogs (3), felines (4), swine (5), guinea pigs (6), ruminants (7), camelids (8), and non-human primates (9).

S. zooepidemicus is a commensal bacteria residing in the skin and mucous membranes of horses. This species is an opportunistic pathogen frequently implicated as the cause of respiratory infections in both foals and adults, as well as genital and urinary tract infections (10). Many commensal bacteria including, *S. zooepidemicus*, colonize the exterior of the stallion penis, but they are not regarded as pathogenic and may be cultured from an ejaculate. However, alterations of the normal bacterial microbiota on the external genitalia may favor the growth of opportunistic bacteria such as *S. zooepidemicus*, which, if inseminated, may cause infertility in susceptible mares (11).

S. zooepidemicus is considered responsible for many emerging zoonotic diseases. *S. zooepidemicus*-associated infections could be considered as zoonoses, since its transmission has been documented by the consumption of unpasteurized milk and dairy products (12), but also due to direct contact with infected horses (13, 14) or infected dogs (15). The possible effects of *S. zooepidemicus* infection in humans include nephritis, arthritis, meningitis, pneumonia, infected aortic aneurysm, infective endocarditis, and sepsis (15).

Antimicrobial resistance is one of the most important threats to global health, food safety, and development. Nowadays, this phenomenon has received much attention due to the potential exposure of humans to resistant bacteria through food chains and direct contact with production and companion animals. Antibiotic resistant strains of *S. zooepidemicus* have been reported in recent studies in Italy (16), United States of America (17), Sweden (18), and Australia (19); however, there is no information about *S. zooepidemicus* and its susceptibility profile to antimicrobials, in Cuban horses. Therefore, the objectives of this study were to determine the occurrence of *S. zooepidemicus* in a horse with pale mucous membranes and its antimicrobial susceptibility profile.

A mare less than two years old, owned by a private producer in Melena del Sur, Mayabeque province, was sampled in 2021. The mare had her vaccination schedule up to date and had not recently been given a treatment with antibiotics, according to the owner. At the time of sample collection, the animal did not present any clinical signs with the exception of pale mucous membranes.

For sampling, the mare's external genitalia were rinsed with physiological saline and then dried. A sample was taken by rotating a sterile swab from the mare's clitoral fossa (20). The swab tip was placed in a tryptone soy agar medium (Merck, Darmstadt, Germany), with 0.5 % bovine albumin. The sample was labeled and transported within 24 hours to the Animal Bacteriology Laboratory of the Department of Animal Health of the National Center for Animal and Plant Health, where it was processed.

The sample was streaked on 5 % sheep blood Columbia agar (VWR, Leuven, Belgium) and incubated aerobically at 37°C for 24 hours. The isolate was identified using Gram staining. Biochemical characterization was assessed through oxidase and catalase tests. The isolate was stored in brain heart infusion (AppliChem, Darmstadt, Germany), with 20 % glycerol at -20 ˚C.

The pinpoint, circular, small, opaque to grayish, and β-hemolytic colonies 1 to 3 mm in diameter were observed. Furthermore, the recovered isolate resulted to be catalase and oxidase negative, and it appeared as Gram-positive cocci organized in chains on the smears. Pathogenic bacteria species are frequently isolated from uterine swabs, uterine lavage, vaginal swabs, and clitoral swabs (20) in mares. *S. zooepidemicus* is usually responsible for a dormant subclinical infection, persisting in endometrium tissue and resisting the immune system and antimicrobial treatment. This is related to *S. zooepidemicus* ability to survive in epithelial cells in the form of "persister" cells (21).

For an initial identification, a biochemically-based commercial system, API 20 Strep (bioMériuex, Marcy L'Etoile, France) was used. For the interpretation of results, the manufacturer's guidelines were followed. API 20 Strep identification result was *Streptococcus equi* subsp. *zooepidemicus* (probability, 99.9%) for the recovered isolate. Then, identification was confirmed by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (MALDI Biotyper®, Bruker Daltonics GmbH & Co.KG, Bremen, Germany). For MALDI-TOF MS identification, fresh colonies were used. Bacterial colonies were directly applied from the culture to the MS plate and left to dry, adding then 1 µL of Bruker Matrix HCCA (Bruker Daltonics GmbH & Co.KG, Bremen, Germany). The identification was based on the score values released by the equipment's instructions. According to Bruker Biotyper´s guidelines, a score value ≥ 2 is interpreted as high-confidence identifications, which means reliable identification at the species level.

S. zooepidemicus isolate was identified at subspecies level with a log (score) of 2.25 by MALDI-TOF MS. *S. zooepidemicus* and *Streptococcus equi* subps. *equi*, the causative agent of strangles in horses, are closely related so they can be misidentified (22). MALDI-TOF MS can be considered a useful, reliable, and rapid technique for *S. zooepidemicus*

identification, able to discriminate between *S. equi* subsp. *equi* (23). Also, Uchida-fujii *et at.* (24) reported that this method is applicable for the identification of equine bacterial isolates. For this reason, in this study, the biochemical API 20 Strep identification of the recovered isolate was confirmed by MALDI-TOF MS analysis.

The antibiotic minimal inhibitory concentration (MIC) was determined using broth microdilution method. The isolate was tested for its susceptibility to 11 antibiotics, belonging to nine different classes: penicillins alone or combined (penicillin G, ampicillin, and amoxicillin-clavulanate) (Sigma-Aldrich, St. Louis, USA), cephalosporins (cefquinome) (Sigma-Aldrich, St. Louis, USA), carbapenems (imipenem) (EDQM CS, Strasbourg Cedex, France), aminoglycosides (gentamicin) (SERVA, Heidelberg, Germany), fluoroquinolones (enrofloxacin) (BioChemika, Espoo, Finland), tetracyclines (doxycycline) (Sigma-Aldrich, St. Louis, USA), macrolides (erythromycin) (SERVA, Heidelberg, Germany), phenicols (chloramphenicol) (SERVA, Heidelberg, Germany), and glycopeptides (vancomycin) (Sigma-Aldrich, St. Louis, USA). Antibiotic choice was performed considering the antibiotics most commonly used in equine clinical practice described in the Clinical and Laboratory Standards Institute (CLSI) manual (25). The broth microdilution method and the stock solutions of antimicrobial agents were performed as described in the CLSI (25) manual.

The isolate was classified as susceptible (S), intermediate (I) or resistant (R), according to both the CLSI (25) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (26) guidelines for *Streptococcus* spp. Breakpoint values to determine susceptibility were used according to EUCAST (26). When reference breakpoints did not exist, in EUCAST (26), CLSI (25), criteria were used (Table 1).

The isolate was susceptible to all *β*-lactam with MIC under 0.25 µg/mL (Table 1). Penicillins are recommended as the first-line of treatment for suspected *S. zooepidemicus* infections because in general, penicillin resistance among *S. zooepidemicus* has remained low (17, 27). However, Nocera *et al.* (16) reported an increase in penicillin and ampicillin resistance, and amoxicillin-clavulanate had the highest intermediate susceptibility value. The trend observed suggests that the susceptibility profile of *S. zooepidemicus* may be less predictable than previously reported, highlighting the importance of culture and susceptibility studies for guiding antimicrobial therapy when possible.

The isolate also presented susceptibility to erythromycin, chloramphenicol, and vancomycin. This result is in concordance with other studies reporting low resistance to macrolides in *S. zooepidemicus* (17, 27). Although resistance to macrolides is low, a temporal increase in macrolide resistance over a longer-term period has been reported. Something similar happened with chloramphenicol, but with higher initial levels of resistance (17). Susceptibility to vancomycin was found in *S. zooepidemicus* strains isolated from humans as well (28).

According to EUCAST Expert Rules (29), *Streptococcus* spp. exhibit low-level intrinsic resistance to aminoglycosides, so these drugs should not be assessed for *S. zooepidemicus* susceptibility. However, the CLSI (25) manual does no report intrinsic resistance to aminoglycosides for streptococci. In addition, several recent studies continue to evaluate the aminoglycoside susceptibility for streptococcal strains (16, 18, 30). The isolate in the present study was resistant to gentamicin with a MIC of 16 µg/mL. There has been an increase in aminoglycoside resistance through time (19). Aminoglycosides have reduced efficacy against streptococci when administered in low concentrations, which is due to their restricted drug uptake (31). However, it has been reported that the combination of aminoglycosides with other antibiotics, such as penicillins and glycopeptides turn out to have a significant bactericidal synergy, especially for internal infection (32).

Table 1. Antimicrobial susceptibility profile of *S. zooepidemicus*. / *Perfil de susceptibilidad a antimicrobianos de* S. zooepidemicus.

Antimicrobial substance	MIC breakpoints values (µg/mL)			Reference	MIC Value (µg/mL)	Interpretation
	S	I	R			
Penicillin G	≤ 0.25	-	> 0.25	(26)	< 0.25	S
Ampicillin	≤ 0.25	-	> 0.25	(26)	< 0.25	S
Amoxicillin - clavulanate	≤ 0.25	-	> 0.25	(26)	< 0.25	S
Cefquinome	≤ 0.25	-	> 0.25	(26)	< 0.25	S
Imipenem	≤ 0.25	-	> 0.25	(26)	< 0.25	S
Gentamicin	≤ 2	4	≥ 8	(25)	16	R
Enrofloxacin	≤ 0.5	-	> 0.5	(26)	2	R
Doxycycline	≤ 1	-	> 1	(26)	2	R
Erythromycin	≤ 0.25	-	> 0.25	(26)	< 0.25	S
Chloramphenicol	≤ 4	8	≥ 16	(25)	4	S
Vancomycin	≤ 1	-	-	(26)	0.5	S

S: susceptible, I: intermediate, R: resistant, "-": not applicable. / *S: susceptible, I: intermedio, R: resistente, "-": no aplicable.*

There was also resistance to enrofloxacin and doxycycline with 2 µg/mL MIC values in both cases. Fluoroquinolones may be an alternative to beta-lactam antibiotics to treat streptococcal infections (32). However, fluoroquinolones, as well as third-generation cephalosporins and higher, are considered by World Health Organization as critically important antimicrobials in human medicine, classified with the priority criterion 1 (33). Therefore, their use in animals should be carried out with caution. Fluoroquinolone resistant strains of *S. zooepidemicus* have been reported in equine, although with a decreasing trend of resistance (16, 17). *S. zooepidemicus* and streptococci often show high levels of resistance to tetracyclines (16). Furthermore, Lord *et al*. (17) reported that the vast majority of isolates (85.3%) were resistant to tetracycline, with significant temporal increase in their study.

A bacterium is considered to be multidrug-resistant if it is non-susceptible to at least one agent from three or more classes of antimicrobials (34, 35). In this study, the isolate was resistance to three antimicrobial classes: aminoglycosides, fluoroquinolones, and tetracyclines. This is in agreement with Lord *et at*. (17), who reported six types of antimicrobial combinations in multiresistant *S. zooepidemicus* strains. In all cases, fluoroquinolones and tetracyclines were part of the combinations. To cope with the alarming increasing circulation of multidrug-resistant strains, empirical equine therapy should be avoided and treatments based on the results of antimicrobial susceptibility testing should be preferred.

Multidrug-resistant *S. zooepidemicus* has been detected for the first time in Cuba. It is a species with pathogenic and zoonotic potential in horses, isolated from a mare genital mucosa. The close interaction between humans and horses increases the risk of acquiring these multiresistant microorganisms or favoring their dissemination, thus this result should be considered in staff training.

Therefore, it is strongly recommended to wash hands with soap and water after contact with horses or other animals. In addition, further studies on the risk factors for its zoonotic transmission and on the spectrum of human diseases associated with *S. zooepidemicus* are needed. The growing rate of antimicrobial resistance represents a serious public health problem with important implications for clinical practice in both human and veterinary medicine.

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