ORIGINAL ARTICLE

Multidrug-resistant bacteria recovered from the respiratory and reproductive tracts of horses from Mayabeque, Cuba

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Bacterias multirresistentes a antimicrobianos recuperadas de los tractos respiratorio y reproductivo de caballos procedentes de Mayabeque, Cuba

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ABSTRACT: Antimicrobial resistance in bacteria isolated from horses represents a threat to global health due to the close relationship of these animals with humans. This study aimed to elucidate the antimicrobial susceptibility profiles of bacteria with pathogenic and zoonotic potential, isolated from the respiratory and genital tracts of horses in Mayabeque, Cuba. Nasal and genital exudates were collected from twenty-three horses, and the isolates obtained were identified using the analytical profile index and mass spectrometry. The minimum inhibitory concentration (MIC) of thirteen antibiotics was determined for the isolates. Twenty-three bacterial isolates, including one *Streptococcus uberis*, sixteen *Enterococcus* spp., three *Staphylococcus sciuri*, one *Morganella morganii*, and two *Stenotrophomonas maltophilia*, were obtained. *S. uberis* showed resistance to gentamicin, enrofloxacin, and doxycycline, while enterococci isolates displayed resistance to doxycycline (n=16), rifampicin (n=8), erythromycin (n=7), enrofloxacin (n=3), and chloramphenicol (n=1). All *S. sciuri* isolates were resistant to at least six antibiotics, including methicillin, with high MIC values for erythromycin (>8192 µg/ml), doxycycline (128 µg/ml), and gentamicin (64 µg/ml). *M. morganii* isolate was resistant to three antibiotics, with MICs of 128 µg/ml and 256 µg/ml for cefquinome and doxycycline, respectively. *S. maltophilia* isolates exhibited resistance to cefquinome, with MIC values of 512 µg/ml and 128 µg/ml, respectively. The 91.3% of the isolates were multiresistant to antimicrobials, which represents the first detection in Cuba of bacteria with multiresistance phenotypes, isolated from samples of the nasal and genital mucosa of horses.

Keywords: Antimicrobial resistance; bacteria; genital mucosa; horses; nasal mucosa.

RESUMEN: La resistencia a antimicrobianos en bacterias aisladas de caballos representa una amenaza para la salud mundial debido al estrecho vínculo de estos animales con los humanos. El objetivo de este estudio fue describir los perfiles de susceptibilidad a antimicrobianos de bacterias con potencial patogénico y zoonótico, aisladas de los tractos respiratorio y genital de caballos procedentes de Mayabeque, Cuba. Se recopilaron exudados nasales y genitales de veintitrés caballos, y se utilizó el índice de perfil analítico y espectrometría de masas para realizar la identificación de los aislados obtenidos. Se determinó la concentración mínima inhibitoria (CMI) de trece antibióticos, frente a los aislados. Se obtuvieron veintitrés aislados bacterianos (un *Streptococcus uberis*, dieciséis *Enterococcus* spp., tres *Staphylococcus sciuri*, un *Morganella morganii* y dos *Stenotrophomonas maltophilia*). *S. uberis* mostró resistencia a gentamicina, enrofloxacina y doxiciclina. Los aislados de enterococci fueron resistentes a doxiciclina (n=16), rifampicina (n=8), eritromicina (n=7), enrofloxacina (n=3) y cloranfenicol (n=1). Todos los aislados de *S. sciuri* fueron resistentes al menos a seis antibióticos incluyendo meticilina, con altos valores de CMI para eritromicina (>8192 µg/ml), doxiciclina (128 µg/ml) y gentamicina (64 µg/ml). El aislado de *M. morganii* fue resistente a tres antibióticos con CMIs de 128 µg/ml y 256 µg/ml para cefquinoma y doxiciclina, respectivamente. Los aislados de *S. maltophilia* mostraron resistencia a cefquinoma con valores de CMI de 512 y 128 µg/ml respectivamente. El 91,3% de los aislados resultaron multirresistentes a antimicrobianos, lo cual representa la primera detección en Cuba de bacterias con fenotipos de multirresistencia, aisladas de muestras de la mucosa nasal y genital de caballos.

Palabras clave: Resistencia a antimicrobianos; bacterias; mucosa genital; caballos, mucosa nasal.

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INTRODUCTION

The excessive and inappropriate use of antimicrobials in both human and veterinary medicine has significantly contributed to the global emergence and dissemination of multidrug-resistant (MDR) bacterial pathogens (1). The surveillance of pathogen resistance in animals plays a crucial role in evaluating the extent and progression of antimicrobial resistance (AMR). According to the WHO's strategic objectives outlined in 2015 (2), surveillance represents the second fundamental goal, aiming to secure the ongoing capability to treat and prevent infectious diseases with effective and safe medications for as long as possible.

AMR challenge is exacerbated by the observed transmission of MDR pathogenic strains from various animal species to humans (3). Horses represent a potential host for the transmission of MDR bacteria to humans (4). Given that horses serve as working animals, pets, and livestock, their close interactions with humans heighten the risk of transmitting antimicrobial-resistant bacteria between the two species (5).

Bacterial infections in horses are often caused by microorganisms considered commensal (6). These microorganisms are the main cause of diseases in the upper and lower respiratory tracts, which often result in reduced performance and exercise intolerance (7). After colonizing the genital tract, bacteria can lead to endometritis, infertility, and abortions in mares, and can be transmitted during mating or insemination (8).

Culture methods for microbial isolation continue to be the "gold standard" for diagnosing infections, despite their low sensitivity. However, conventional methods are time-consuming and often inadequate for identifying phenotypically similar species. For this reason, traditional diagnostic methods need to be complemented with the use of molecular analysis techniques (9). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) technique enables the discrimination between two bacterial subspecies with high specificity (10, 11). Current DNA sequencing technologies hold the potential to develop AMR diagnostic tools capable of providing rapid information within hours, rather than days. Nonetheless, reliable AMR diagnosis necessitates phenotypic tests where bacteria are exposed to antimicrobials. The most commonly employed techniques for determining the antimicrobial susceptibility profile of bacteria are Kirby-Bauer agar diffusion or broth microdilution (12).

Several studies have been conducted to elucidate the evolution and prevalence of AMR in pathogens isolated from horses in various countries (6, 7, 13). In Cuba, research has been focused on studying pathogens affecting horses (14-16). However, surveillance of AMR in bacteria recovered from clinical samples of this animal species is not conducted. Therefore, the objective of this study was to describe antimicrobial susceptibility profiles of potentially pathogenic and zoonotic bacteria isolated from the respiratory and genital tracts of horses from Mayabeque, Cuba.

MATERIALS AND METHODS

Sampling

Horses from Mayabeque province, which had a total of 21,112 specimens during the study period, were included. Two convenience samplings were carried out in Melena del Sur and Quivicán municipalities, in Mayabeque province, Cuba, in April and May 2021, respectively. These samplings were carried out as part of the vaccination program against equine infectious anemia, focusing specifically on the areas near the southern coast, where there is the highest risk of disease transmission. The horses were owned by private producers in these areas, and the owners reported that the animals were used for agricultural work and transportation. At the time of sampling, horses did not exhibit any clinical signs of illness. The study was approved by the Animal Research Ethics Committee of the National Center of Animal and Plant Health (CENSA), as part of the "Strengthening diagnostic capabilities for pathogens of interest in equines" project. Sampling was authorized by the National Center for Animal Health (CENASA) of the Ministry of Agriculture (MINAG).

Twenty-three animals (ten from Melena del Sur and thirteen from Quivicán) were sampled. Nasal and genital swab samples were collected from all the animals using sterile swabs. Nasal swabs were obtained from inside horse nostrils. For mares, the genital sample was collected by a swab in the clitoral fossa, while for males, a preputial swab was taken. Prior to sample collection, nostrils and external genitalia of horses were rinsed with sterile physiological saline, and then dried (17).

The swab tip was placed in 0.5% bovine albumin-supplemented tryptone soy agar (Merck, Darmstadt, Germany). After labeling, samples were stored in thermal containers to maintain a temperature range from 4 - 8 °C. They were then transported to the Animal Bacteriology Laboratory of the Animal Health Department at CENSA within 4 hours for initial processing.

Bacteriological Isolation

The samples collected were streaked on 5 % sheep blood Columbia agar (VWR, Leuven, Belgium) and incubated aerobically at 37 °C for 24 hours. The selection of colonies resulting from the primary culture was based on the predominance of growth in the second or third streak. In cases where multiple phenotypes coexisted, the predominant one was chosen, ensuring one phenotype per sample. Subsequently, subculturing was carried out, and isolates were preliminarily identi-

fied using Gram staining (Thermo Fisher Scientific, Massachusetts, USA). Additionally, the isolates were characterized through culture on MacConkey agar (Oxoid, Basingstoke, United Kingdom), catalase tests (Sigma-Aldrich, St. Louis, USA), and oxidase tests (Sigma-Aldrich, St. Louis, USA). All procedures were conducted following the manufacturers' instructions for the techniques.

Isolates were then stored in brain heart infusion broth (AppliChem, Darmstadt, Germany) supplemented with 20 % glycerol at -20°C, and also stabbed into nutrient agar (BioCen, Bejucal, Cuba) at 4°C, for transport to the Epidemiology and Preventive Medicine Laboratory of the University Institute of Animal Health and Food Safety in Gran Canaria, Spain.

Phenotypic Identification

The biochemical test kits API 20 Strep, API Staph, and API 20 E (bioMérieux, Marcy L'Étoile, France) were used for initial identification based on the suggestive genus type reported in the preliminary identification. Manufacturer's recommendations were followed for conducting the assays and interpreting the results. Probability values above 90 % and 80 % were considered excellent and with good levels of identification, respectively, while values below 60 % indicated unreliable identification.

Confirmatory Identification

The isolates underwent confirmatory identification through MALDI-TOF MS analysis (MALDI Biotyper®, BrukerDaltonics GmbH and Co.KG, Bremen, Germany). Fresh bacterial colonies were directly applied to the MS plate and allowed to dry. Subsequently, 1 µl of Bruker Matrix HCCA (α-cyano-4-hydroxycinnamic acid) was added. Identification was based on the score values provided by the equipment's instructions. According to Bruker Biotyper guidelines, score values ≥ 2 were interpreted as high-confidence identifications, indicating reliable species-level identification.

Antimicrobial Susceptibility Testing

The MIC of the isolates was determined using the broth microdilution method, following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) manual (18). The antibiotics commonly used in equine clinical practice, as described in CLSI manual (18), were selected. Mother solutions of the antimicrobial agents and dilutions were prepared following the procedures described in CLSI manual (18).

Thirteen antibiotics from ten different classes were used to determine the susceptibility of the isolates. Antibiotics included penicillins alone or in combination (penicillin G, methicillin, ampicillin, amoxicillin-clavulanic acid) (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), ansamycins (rifampicin) (Sigma-Aldrich, St. Louis, USA), and glycopeptides (vancomycin) (Sigma-Aldrich, St. Louis, USA).

A standard ninety-six-well round-bottom plate (Thermo Fisher Scientific, Massachusetts, USA) was used for antimicrobial susceptibility testing, allowing simultaneous testing of eight antibiotics in ten two-fold serial dilutions on a single plate. Antibiotics were dissolved and diluted to create working solutions in Mueller-Hinton broth cation adjusted (MHBCA) (Oxoid, Basingstoke, United Kingdom). Subsequently, 100 µL of diluted antibiotics in MHBCA were added to the first row of wells, and nine double serial dilutions were carried out in the remaining rows, extending to row ten. Rows eleven and twelve were used as positive and negative controls, respectively, with no antibiotic applied to the positive control wells.

Bacterial suspensions in saline solution were prepared from agar plate growths of each isolate, and their turbidity was adjusted to 0.5 on the McFarland scale using a barium chloride standard. These bacterial suspensions contained approximately 1.00 x 10^8 CFU/ml, and then they were diluted in MHBCA to 1.00 x 10^6 CFU/ml. Subsequently, 100 µL of the suspension of each bacterial isolate were inoculated into each well of the microdilution plate, except in negative control wells. After 24 hours of incubation at 37 °C, MIC was determined by evaluating the turbidity or the presence of bacterial growth at the bottom of the well. MIC determination of each antibiotic for each isolate was performed in triplicate.

The isolates were categorized as susceptible (S), intermediate resistant (I), or resistant (R), in accordance with CLSI guidelines (18). The susceptibility cut-off points were determined following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) manual (19). In instances where specific cut-off values were not provided by EUCAST, the values outlined in CLSI manual (18) were utilized. If veterinary reference cut-offs were unavailable, the criteria specified in CLSI manual for human use (20) were employed. Isolates were deemed MDRs if they demonstrated non-susceptibility to at least one agent from three or more classes of antimicrobials (21).

RESULTS AND DISCUSSION

Origin and identification of isolates

Twenty-three bacterial isolates, including one *Streptococcus uberis*, sixteen *Enterococcus* spp. (seven *Enterococcus casseliflavus*, six *Enterococcus*

faecium, and three *Enterococcus mundtii*), three *Staphylococcus sciuri*, one *Morganella morganii*, and two *Stenotrophomonas maltophilia*, were obtained. The identification results obtained through API and MALDI-TOF MS tests are summarized in Table 1.

The identifications obtained by API and MALDI-TOF MS coincided in all cases except for isolates 13A, 17A, 25A, 46A, 58A, 73A, 75A, 97A, 104, and 114A. Whenever *E. faecium* is identified, the API 20 Strep manufacturer recommends additional testing to confirm identification. MALDI-TOF MS technique is described as useful, reliable, and rapid for the identification of equine bacteria, capable of discriminating among the isolates that are difficult to identify with biochemical methods (11,22). Therefore, in cases where identifications did not match, the result obtained by MALDI-TOF MS technique was chosen to name the species to which the isolates belonged.

Regarding Table 1, it presents the type of exudate collected and the sex of the animals sampled. Only *Enterococcus* spp. and *Staphylococcus* spp. were found in samples from males. In contrast, all five genera were identified in samples obtained from females. *Enterococcus* spp., *Staphylococcus* spp., and *Stenotrophomonas* spp. were isolated from nasal exudates, while genital exudates contained all the genera described in this study, except *Stenotrophomonas* spp. Respiratory and reproductive infections cause significant losses in the equine industry, thus it is important to conduct studies focused on diagnosing and characterizing the bacteria responsible for these conditions (6), although the animals considered in this study did not show clinical signs.

Gram-positive Bacteria

The presence of three different genera of Gram-positive bacteria in this study, namely *Streptococcus* spp., *Enterococcus* spp., and *Staphylococcus* spp., aligns with previous findings linking these genera to contagious diseases that pose a threat to equine health (23).

Streptococcus uberis

S. uberis is commonly isolated from clinical cases of mastitis in cattle (10) and has also been detected in the blood of foals with intravenous catheters inserted

Table 1. Identification results of isolates recovered from horse exudate samples, Mayabeque 2021, according to API and MALDI-TOF MS tests. / *Resultados de la identificación de los aislados recuperados de muestras de exudados de caballos, Mayabeque 2021, según pruebas API y MALDI-TOF MS.*

Isolate code	Identification according to API	Identification according to MALDI-TOF MS	Type of exudate	Sex of the animal	Place of origin
145A	*Streptococcus uberis*	*Streptococcus uberis*	g	f	Q
13A	*Enterococcus faecium*	*Enterococcus casseliflavus*	n	m	M
17A	*Enterococcus faecium*	*Enterococcus casseliflavus*	n	m	M
25A	*Enterococcus faecium*	*Enterococcus casseliflavus*	n	m	M
46A	*Enterococcus faecium*	*Enterococcus casseliflavus*	n	f	M
58A	*Enterococcus faecium*	*Enterococcus casseliflavus*	g	m	M
73A	*Enterococcus faecium*	*Enterococcus casseliflavus*	g	f	M
75A	*Enterococcus faecium*	*Enterococcus casseliflavus*	g	f	M
27A	*Enterococcus faecium*	*Enterococcus faecium*	n	m	M
56A	*Enterococcus faecium*	*Enterococcus faecium*	g	m	M
130	*Enterococcus faecium*	*Enterococcus faecium*	g	f	Q
153A	*Enterococcus faecium*	*Enterococcus faecium*	g	f	Q
161	*Enterococcus faecium*	*Enterococcus faecium*	g	f	Q
163A	*Enterococcus faecium*	*Enterococcus faecium*	g	f	Q
97A	*Enterococcus faecium*	*Enterococcus mundtii*	n	f	Q
104	*Enterococcus faecium*	*Enterococcus mundtii*	n	m	Q
114A	*Enterococcus faecium*	*Enterococcus mundtii*	n	f	Q
110C	*Staphylococcus sciuri*	*Staphylococcus sciuri*	n	f	Q
129B	*Staphylococcus sciuri*	*Staphylococcus sciuri*	g	m	Q
158A	*Staphylococcus sciuri*	*Staphylococcus sciuri*	g	m	Q
50B	*Morganella morganii*	*Morganella morganii*	g	f	M
90C	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*	n	f	Q
93A	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*	n	f	Q

n: nasal, g: genital, f: female, m: male, M: Melena del Sur, Q: Quivicán. / *n: nasal, g: genital, f: hembra, m: macho, M: Melena del Sur, Q: Quivicán.*

for the treatment of medical or surgical conditions (13). It is an opportunistic pathogen capable of adapting to various ecological niches due to its nutritional flexibility. Sherwin *et al*. (24) demonstrated *S. uberis*' ability to survive for at least 35 days in straw and sand bedding substrates, highlighting its potential for replication and the challenges associated with its control.

S. uberis exhibited susceptibility to all *β*-lactams, erythromycin, and vancomycin, with MIC values below 0.25 µg/ml. However, it was resistant to gentamicin, enrofloxacin, and doxycycline. The number of *S. uberis* isolates obtained from horses is limited, thus the information used here was sourced from studies analyzing strains isolated from cases of mastitis in cows.

In the present study, *S. uberis* displayed resistance to gentamicin with a MIC of 32 µg/ml. Rosa *et al*. (25) reported a high incidence of aminoglycoside resistance in *S. uberis*. Elevated levels of gentamicin resistance have been observed in *Streptococcus* spp. isolates due to the presence of the bifunctional aminoglycoside-inactivating enzyme 6′-acetyltransferase-2′′-phosphotransferase [AAC(6′)-APH(2′′)] encoded by the *aacA-aphD* gene (26).

Furthermore, *S. uberis* displayed resistance to enrofloxacin and doxycycline with MIC values of 1 and 8 µg/ml, respectively. Streptococci commonly exhibit significant resistance to tetracyclines (27). Variable rates of tetracycline-resistant *S. uberis* isolates, ranging from 45 % to 100 %, have been reported by Zhang *et al*. (28). This antibiotic is more likely to be excreted slowly from the body over an extended period. Its low degradative nature leads to an increase in selective pressure, potentially resulting in microbial resistance typically encoded by plasmids and/or transposable elements (29).

In the present study, *S. uberis* demonstrated resistance to three classes of antimicrobials: aminoglycosides, fluoroquinolones, and tetracyclines. Previous reports (10) have indicated the prevalence of MDR *S. uberis* isolates at 94 % in milk from cows with clinical mastitis. Aminoglycosides and tetracyclines have been identified as the primary antibiotics associated with the multidrug resistance pattern observed in *S. uberis* (10,25).

Enterococcus spp.

E. faecium and *E. faecalis*, among the *Enterococcus* species, are the primary contributors to various diseases. They have become etiological agents of the most common nosocomial infections and pose an increasing threat to public health due to their intrinsic resistance to multiple antimicrobials. Reports have shown antimicrobial-resistant *E. faecium* in the feces of foals and racehorses (22). *E. casseliflavus* is an opportunistic pathogen that primarily affects immunocompromised individuals or those with chronic diseases, typically acquired in nosocomial environments (30). It has been implicated in a case of septic meningitis in a foal (31) and as the causative agent of endometritis in a mare (32). Antimicrobial-resistant *E. mundtii* isolates have been identified in vaginal samples from mares (33).

Among the sixteen enterococci isolates, the highest resistance rate was observed for doxycycline (100 %; 16/16), followed by rifampicin (50 %; 8/16), erythromycin (43.75 %; 7/16), enrofloxacin (18.75 %; 3/16), and chloramphenicol (6.25 %; 1/16) (Table 2). All isolates were susceptible to ampicillin and vancomycin. Anyanwu *et al*. (34) found high levels of resistance to rifampicin (90 %), erythromycin (80 %), and chloramphenicol (36.7 %), which is consistent with the findings of this study. These resistances likely result from acquired genes under the selection pressure of antimicrobial use.

Table 2. Distribution of minimum inhibitory concentrations (MICs) (µg/ml) and resistance of the sixteen *Enterococcus* spp. isolates (seven *Enterococcus casseliflavus*, six *Enterococcus faecium*, and three *Enterococcus mundtii*) recovered from horse exudate samples, Mayabeque 2021. The results are shown as the percentage of isolates at different MIC values. / *Distribución de las concentraciones mínimas inhibitorias (CMIs) (µg/ml) y la resistencia de los dieciséis aislados de* Enterococcus *spp. (siete* Enterococcus casseliflavus*, seis* Enterococcus faecium *y tres* Enterococcus mundtii*) recuperados de muestras de exudados de caballos, Mayabeque 2021. Los resultados se muestran como el porcentaje de aislados en los diferentes valores de CMI.*

Antibiotics	n	< 0,25	0,5	1	2	4	8	16	32	64	128
Ampicillin	16		31,3 (5)	43,8 (7)	25,0 (4)						
Enrofloxacin	16				81,3 (13)	12,5 (2)			6,3 (1)		
Doxycycline	16					50,0 (8)	37,5 (6)	12,5 (2)			
Erythromycin	16	6,3 (1)	12,5 (2)	18,8 (3)	6,3 (1)	12,5 (2)	12,5 (2)	25,0 (4)	6,3 (1)		
Chloramphenicol	16						12,5 (2)	81,3 (13)		6,3 (1)	
Rifampicin	16	37,5 (6)			12,5 (2)	37,5 (6)	6,3 (1)			6,3 (1)	
Vancomycin	9			100,0 (9)							

n: number of isolates assessed. Vertical lines indicate the cut-off values used to define intermediate resistance (black) and resistance (red). The values between parentheses represent the number of isolates. / *n: cantidad de aislados evaluados. Las líneas verticales indican los puntos de corte usados para determinar resistencia intermedia (negro) y resistencia (rojos). Los valores entre paréntesis representan la cantidad de aislados.*

Enterococcus spp. exhibit intrinsic resistance to several classes of antimicrobials (18), including cephalosporins and aminoglycosides, posing challenges in the selection of appropriate antimicrobials for their control. In line with the findings of this study, Kim *et al*. (35) documented a high prevalence of tetracycline resistance among enterococcal strains, reaching 50 %. This resistance trend was linked to the extensive use of tetracyclines in veterinary medicine in Korea.

Eight of the total *Enterococcus* spp. isolates exhibited resistance to rifampicin, with MIC values significantly exceeding the cut-off point. A study conducted in 2003 by the SENTRY antimicrobial surveillance program revealed rifampicin resistance in 65.9 % of vancomycin-resistant *E. faecium* isolates in the U.S. and 67.5 % in Europe (36). Although, in the present study, all isolates were susceptible to vancomycin.

Table 3 illustrates the MDR patterns of the *Enterococcus* spp. isolates of this study. MDR *Enterococcus* spp. isolates with resistance to tetracyclines, macrolides, fluoroquinolones, and phenicols have been documented in horses (22), consistent with the majority of MDR patterns described in the *Enterococcus* spp. isolates in this study. The acquisition of external resistance genes in *Enterococcus* spp. is often associated with the transfer of plasmids carrying antibiotic resistance genes. Plasmids bestowing resistance to vancomycin, macrolides, tetracycline, aminoglycosides, and heavy metals (such as copper, cadmium, zinc bacitracin) have been identified on farms exposed to antimicrobials used as growth promoters (e.g., avoparcin, virginiamycin, tylosin, or zinc bacitracin), for therapeutic purposes (e.g., tetracyclines, gentamicin, penicillins), or as dietary supplements (e.g., copper) (37).

Table 3. Multidrug resistance patterns of *Enterococcus* spp. isolates recovered from horse exudate samples, Mayabeque 2021. / *Patrones de multirresistencia de los aislados de* Enterococcus *spp. recuperados de muestras de exudados de caballos, Mayabeque 2021.*

Isolate code	Multidrug-resistance patterns
13A, 17A, 25A, 27A, 58A, 73A, 75A	ENR-DOX-ERY-CHL-RIF
46A	ENR-DOX-CHL-RIF
56A, 130	ENR-DOX-ERY-RIF
104, 153A, 161, 163A	ENR-DOX-ERY-CHL
97A, 114A	ENR-DOX-CHL

ENR: enrofloxacin, DOX: doxycycline, ERY: erythromycin, CHL: chloramphenicol, RIF: rifampicin. / *ENR: enrofloxacina, DOX: doxiciclina, ERY: eritromicina, CHL: cloranfenicol, RIF: rifampicina.*

Staphylococcus sciuri

S. sciuri is an opportunistic pathogen found in a variety of habitats, including animals, humans, and the environment. This bacterium is a significant human pathogen and can be responsible for a range of conditions such as endocarditis, peritonitis, urinary tract infections, wound/skin infections, and septic shock (38). In animals, *S. sciuri* has been known to cause fatal exudative epidermitis in piglets, wound infections in horses, and mastitis in cattle. Notably, *S. sciuri* is frequently isolated from both healthy and infected horses, and at times, from stable personnel. Consequently, humans may also contribute to the transmission of these bacteria (39).

All *S. sciuri* isolates analyzed were resistant to at least six of the antibiotics studied (Table 4), with the following concentration ranges: methicillin (8 µg/ml and 16 µg/ml), amoxicillin-clavulanate (0.5 µg/ml and 1 µg/ml), gentamicin (64 µg/ml), enrofloxacin (1 µg/ml), doxycycline (64 µg/ml and 128 µg/ml), and erythromycin (> 8192 µg/ml). Two isolates exhibited resistance to penicillin (0.5 µg/ml and 1 µg/ml) and ampicillin (0.5 µg/ml), while one isolate was resistant to cefquinome (4 µg/ml). All isolates showed intermediate resistance to chloramphenicol and susceptibility to rifampicin and vancomycin.

The *S. sciuri* isolates in this study showed remarkably high MIC values, particularly against erythromycin. The three primary resistance mechanisms to macrolides in staphylococci include bacterial ribosome modification, macrolide efflux from the bacterial cell/ribosome protection through ABC family proteins, and enzymatic inactivation (40). The presence of these three resistance mechanisms in the *S. sciuri* isolates could account for the elevated MIC values against erythromycin. It is imperative to carry out studies employing molecular tools to unravel the genetic basis underlying the observed phenotypic resistance manifestations (12).

The three *S. sciuri* isolates in this study were resistant to methicillin. Methicillin resistance in *Staphylococcus* spp. is determined by the *mecA* or *mecC* gene, located on the bacterial chromosome, and is part of the region known as SCC*mec* (Staphylococcal Chromosomal Cassette *mec*). Both *mecA* and *mecC* are responsible for the synthesis of a modified protein that prevents the binding of penicillins, cephalosporins (except for the latest generation), carbapenems, or monobactams (41). Methicillin-resistant *S. sciuri* has been reported in both healthy (39) and diseased horses (42). Additionally, *S. sciuri* was the most prevalent methicillin-resistant isolate (66.7%, n=12/18) in nasal exudates from healthy racehorses, as per Fungwithaya *et al*. (38). Methicillin resistance rates can be extremely high even in bacteria isolated from healthy animals in the community (43).

In the present study, the *S. sciuri* isolates showed MDR phenotypes (Table 5). Various methicillin-resistant CoNS species exhibit multidrug resistance, acting as reservoirs for resistance genes (39). Methicillin-resistant *Staphylococcus* spp. strains often carry addi-

Table 4. Distribution of minimum inhibitory concentrations (MICs) (µg/ml) and resistance of the three *Staphylococcus sciuri* isolates recovered from horse exudate samples, Mayabeque 2021. The results are shown as the percentage of isolates at different MIC values. / *Distribución de las concentraciones mínimas inhibitorias (CMIs) (µg/ml) y la resistencia de los tres aislados de* Staphylococcus sciuri *recuperados de muestras de exudados de caballos, Mayabeque 2021. Los resultados se muestran como el porcentaje de aislados en los diferentes valores de CMI.*

Antibiotics	n	< 0,25	0,5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	> 8192
Penicillin	3	33,3 (1)	33,3 (1)	33,3 (1)													
Methicillin	3						33,3 (1)	66,7 (2)									
Ampicillin	3	33,3 (1)	66,7 (2)														
Amoxicillin / clavulanate	3		33,3 (1)	66,7 (2)													
Cefquinome	3	33,3 (1)			33,3 (1)	33,3 (1)											
Gentamicin	3									100,0 (3)							
Enrofloxacin	3		66,7 (2)	33,3 (1)													
Doxycycline	3									33,3 (1)	66,7 (2)						
Erythromycin	3																100,0 (3)
Chloramphenicol	3							100,0 (3)									
Rifampicin	3	100,0 (3)															
Vancomycin	3				100,0 (3)												

n: number of isolates assessed. Vertical lines indicate the cut-off values used to define intermediate resistance (black) and resistance (red). The values between parentheses represent the number of isolates. / *n: cantidad de aislados evaluados. Las líneas verticales indican los puntos de corte usados para determinar resistencia intermedia (negro) y resistencia (rojos). Los valores entre paréntesis representan la cantidad de aislados.*

tional resistance genes for sulfonamides, gentamicin, kanamycin, macrolides, fluoroquinolones, or tetracyclines (41). The SCC*mec*, responsible for methicillin resistance, may also contain regions encoding resistance to other classes of antibiotics (44). Besides, bacteria can develop resistance by expelling antibiotics, with the overexpression of efflux pumps being the primary cause of resistance to multiple drugs (45).

Gram-negative bacteria

Two types of Gram-negative bacteria, belonging to the genera *Morganella* and *Stenotrophomonas*, were identified in this study. These genera are recognized for their high intrinsic rates of antimicrobial resistance (18), presenting a challenge for effective antimicrobial therapy and posing a risk to health. Given the limited reports of *M. morganii* and *S. maltophilia* in animals, the studies mentioned below pertain to isolates obtained from humans.

Morganella morganii

M. morganii may act as a reservoir of resistance genes, facilitating its transfer to bacteria via plasmids or other carriers, leading to widespread dissemination of antimicrobial resistance (46). Intrinsic resistance to multiple drugs and increasing infection rates pose a significant risk of *M. morganii* evolving into the next "superbug." These microorganisms are particularly worrisome due to their capacity to evade treatment and cause mortality in their hosts, resulting in substantial costs for individuals and society (47).

In this study, the *M. morganii* isolate was found to be resistant to cefquinome with an MIC of 128 µg/ml. Cefquinome, a fourth-generation cephalosporin used in veterinary medicine, is known to display antibacterial activity against pathogens isolated from horses (48). However, due to the high effectiveness and critical importance of cephalosporins in human medicine (49), their administration in animals should be based on antimicrobial susceptibility testing to minimize the risk of developing cephalosporin-resistant strains that could potentially be transmitted to humans.

The use of carbapenems is typically recommended to treat *M. morganii* infections. These antimicrobials are primarily intended for use in human medicine within hospital settings and are seldom used in horses (50). However, in this particular study, the *M. morganii* isolate demonstrated resistance to imipenem (16 µg/ml), suggesting a potential transfer of resistant bacteria from humans to horses.

In the present study, the *M. morganii* isolate showed susceptibility to gentamicin, enrofloxacin, and chloramphenicol, with MIC values of 2, less than 0.25, and 8 µg/ml, respectively. This aligns with the findings of Zaric *et al*. (46), who noted that gentamicin was the most commonly used antibiotic in treating *M.*

Table 5. Multidrug resistance patterns of *Staphylococcus sciuri* isolates recovered from horse exudate samples, Mayabeque 2021. / *Patrones de multirresistencia de los aislados de* Staphylococcus sciuri *recuperados de muestras de exudados de caballos, Mayabeque 2021.*

Isolate code	Multidrug-resistance patterns
129B	PEN-MET-AMP-AMC-CEQ-GEN-ENR-DOX-ERY-CHL
158A	PEN-MET-AMP-AMC-GEN-ENR-DOX-ERY-CHL
110C	MET-AMC-GEN-ENR-DOX-ERY-CHL

PEN: penicillin, MET: methicillin, AMP: ampicillin, AMC: amoxicillin-clavulanic acid, CEQ: cefquinome, GEN: gentamicin, ENR: enrofloxacin, DOX: doxycycline, ERY: erythromycin, CHL: chloramphenicol. / *PEN: penicilina, MET: meticilina, AMP: ampicilina, AMC: amoxicilina - clavulanato, CEQ: cefquinoma, GEN: gentamicina, ENR: enrofloxacina, DOX: doxiciclina, ERY: eritromicina, CHL: cloranfenicol.*

morganii infection (n = 15; 25%). Their study also indicated frequent use of ciprofloxacin (n = 10; 16%) and amikacin (n = 8; 13%), suggesting a trend towards susceptibility to these antibiotics.

The global dissemination of MDR *M. morganii* strains is on the rise, with recent identification of mobile integrative elements in *M. morganii* isolates, including the multidrug-resistant Salmonella genomic island 1 (SGI1). In the current study, the *M. morganii* isolate exhibited resistance to doxycycline (MIC = 256 µg/ml), potentially linked to the presence of tetracycline resistance genes such as *tet*(B), encoding efflux pumps in Gram-negatives (51), or by the presence of the previously described SGI1variant.

Stenotrophomonas maltophilia

S. maltophilia, an opportunistic bacterial pathogen from environmental origin, is known for causing infections, particularly in hospital settings. This bacterium produces biofilms and virulence factors that promote colonization or infection in susceptible hosts (52). In addition to its impact on human health, *S. maltophilia* can also act as a respiratory pathogen in horses (53). Owing to its intrinsic resistance, the options for controlling *S. maltophilia* are limited to certain classes of antimicrobials, such as cephalosporins, fluoroquinolones, tetracyclines, and phenicols (20).

S. maltophilia exhibits intrinsic resistance to first and second-generation cephalosporins, but not to more recent generations of cephalosporins (20). As a result, a determination of the MIC of the isolates under study against cefquinome was adequate. The isolates were found to be resistant to cefquinome, with MIC values of 128 µg/ml and 512 µg/ml, respectively (Table 6).

Acquired resistance to cephalosporins in *S. maltophilia* is associated with amino acid substitution mutations in SmeH, the transporter protein of the RND efflux pump SmeGH family. These mutations confer resistance to ceftazidime, a third-generation cephalosporin, and other *β*-lactam drugs in *S. maltophilia* strains. The development of resistance in *S. maltophilia* related to RND efflux pumps is also linked to mutations in its regulatory genes, which stimulate its overexpression (54).

Minocycline, doxycycline, and tigecycline are among the most potent antimicrobial agents against *S. maltophilia* (55). However, in this study, one *S. maltophilia* isolate was resistant to doxycycline (16 µg/ml), while another exhibited intermediate resistance (8 µg/ml). The overexpression of SmeDEF is the common genetic determinant of doxycycline resistance. A mutation in the regulatory gene *smeT*, such as the A/T mutation at position 498, leads to overexpression of smeDEF and multiple resistance to tigecycline, doxycycline, and levofloxacin (56).

Table 6. Distribution of minimum inhibitory concentrations (MICs) (µg/ml) and resistance of the two *Stenotrophomonas maltophilia* isolates recovered from horse exudate samples, Mayabeque 2021. The results are shown as the percentage of isolates at different MIC values. / *Distribución de las concentraciones mínimas inhibitorias (CMIs) (µg/ml) y la resistencia de los dos aislados de* Stenotrophomonas maltophilia *recuperados de muestras de exudados de caballos, Mayabeque 2021. Los resultados se muestran como el porcentaje de aislados en los diferentes valores de CMI.*

Antibiotics	n	< 0,25	0,5	1	2	4	8	16	32	64	128	256	512
Cefquinome	2										50,0 (1)		50,0 (1)
Enrofloxacin	2	100,0 (2)											
Doxycycline	2						50,0 (1)	50,0 (1)					
Chloramphenicol	2					50,0 (1)	50,0 (1)						

n: number of isolates assessed. Vertical lines indicate the cut-off values used to define intermediate resistance (black) and resistance (red). The values between parentheses represent the number of isolates. / *n: cantidad de aislados evaluados. Las líneas verticales indican los puntos de corte usados para determinar resistencia intermedia (negro) y resistencia (rojos). Los valores entre paréntesis representan la cantidad de aislados.*

There are no official reports on the use and regulation of antibiotics in Cuban horses. The findings of this study suggest that these horses may have been previously subjected to antibiotic treatment. The limited availability of antimicrobials and restricted access to veterinary services contribute to inadequate and incomplete administration of antibiotics by horse owners and non-professionals without veterinary oversight. Unfortunately, no prior assessment was carried out to determine the potential use of antibiotics in these Cuban horses.

This pioneering study in Cuba has focused on monitoring potentially pathogenic and zoonotic bacteria exhibiting multidrug resistance in horses. Analysis of nasal and genital mucosa samples from horses revealed twenty-three antibiotic-resistant bacterial isolates. This research underscores the role of companion animals as reservoirs of MDR bacteria, emphasizing the looming threat of untreatable infections. Despite the limitation posed by the modest sample size, the study paves the way for methodological advancements in efforts to combat AMR in horse breeding in Cuba. It is imperative to formulate national guidelines governing the use of antimicrobials in the treatment of companion animals and to establish more robust surveillance systems to effectively address this issue.

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