










Antibiotic Resistance in *Enterobacteriaceae* Family Members Isolated from Horses Used for Animal Traction

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Abstract

Lately, bacterial drug resistance has become an important worldwide problem in one health, where bacteria have undergone mutation becoming increasingly resistant. The major problem of bacterial drug resistance is the difficulty with eliminating microorganisms from different wounds and infected patients, and the therapeutic option is most often ineffective as a result of the repeated and inappropriate use of antimicrobials. The objective of this work was to detect and identify enterobacteria, to evaluate their resistance profile and the production of extended-spectrum β -lactamases in draft horse isolates from the municipality of Umuarama, Parana, Brazil. A nasal, oral, and ear cavity swab was collected from 38 horses (used for animal traction) for isolation and bacterial identification, phenotypic antibiotic susceptibility testing, and the phenotypic test for the detection of extended-spectrum β -lactamases-producing strains. In 12 swabs bacterial isolation was possible. Strains of *Escherichia coli*, *Serratia rubidaea*, *Citrobacter diversus*, *Kluyvera* species, and *Providencia alcalifaciens* were isolated. One hundred percent multidrug resistance was detected, and the antimicrobials that encountered the highest resistance were ertapenem (100%), cefotaxime (100%), ceftiofur (100%), ampicillin (100%), amoxicillin (100%), chloramphenicol (100%), and aztreonam (91.67%) and no extended-spectrum β -lactamases -producing strain was detected. The results of this work reveal the presence of strains of the Enterobacteriaceae family associated with high bacterial resistance in horses used for animal traction in the municipality of Umuarama, State of Parana, Brazil, and these results confirm that these horses can be considered reservoirs of multidrug-resistant microorganisms. This situation can be considered an important problem of one health.

Keywords: Antibiotics, Antimicrobials, Bacteria, Horses, One Health

INTRODUCTION

Antimicrobials are natural, synthetic, or semisynthetic substances that have the ability to prevent the development of microorganisms, being used worldwide in the treatment and prophylaxis of different infectious diseases prescribed for both human and animal use (Arias and Carrilho, 2012). They can be classified as bactericidal, when they promote bacterial death, or bacteriostatic, when they inhibit bacterial growth, hence, preventing the increase in the number of microbial cells (Arias and Carrilho, 2012).

The large-scale use of antibiotics in outpatient clinics, hospitals, and at home (self-medication) has affected microbial ecology, which has favored the development of bacterial drug resistance. This is currently a complex problem because selective pressure favors the naturally drug-resistant microbes and mutations can occur (spontaneous or not) and different strains may share genetic material (conjugation) (Loureiro et al., 2016).

Oliveira et al. (2010) stated that starting an antimicrobial treatment only by means of evidence, without analyzing in detail the clinical and laboratory data (microbiological culture for determination of bacterial sensitivity *in vitro*), may favor the development of bacterial drug

resistance and consequently contribute to the therapeutic ineffectiveness in both humans and animals. Another situation that draws attention is the possibility of indirect transfer of resistance genes between bacteria present in the human and animal microbiota and vice versa (Pansani et al., 2016).

Large animals such as horses can also be a source of dissemination of drug-resistant microorganisms owing to the high bacterial diversity and consequently drug resistance allowing for the transmission of this trait to other animals, and even to humans, through the different management schemes on a farm, exposure to water, pastures, and possibly contaminated fomites (Kirinus et al., 2011).

In horse species, studies have demonstrated the presence of multidrug-resistant and extended-spectrum β -lactamase (ESBL)-producing strains from the family *Enterobacteriaceae* (Aguiar et al., 2005; Oliveira et al., 2007; Silva and Lincopan, 2012; Carneiro et al., 2017). Hence, the objective of this work was to detect and identify enterobacteria and to evaluate their drug resistance profile and the production of ESBLs in draft-horse isolates from the municipality of Umuarama, Parana, Brazil.

MATERIAL AND METHODS

Location of the Study and Study Population

In the period from March to September 2016, swabs of the nasal, oral, and ear cavities were collected from mestizo horses of both sexes, over 2 years of age used for traction on different small rural properties of family farming in the city of Umuarama, Parana. At the time of collection, the animals presented no clinical symptoms for any disease.

Sample Collection

Samples were collected using sterile swabs containing AIMES + activated carbon (Copan Transystem®, Italy), which were introduced into the respective cavity (nasal, oral, and ear) and circular and rotational movements were performed in each region. All the samples were kept and refrigerated in the Laboratory of Preventive Veterinary Medicine and Public Health of the Graduate Program in Animal Science with concentration on Bioactive Products at the Paranaense University (UNIPAR).

Bacterial Isolates

Swabs containing the nasal, oral, and ear microbe samples were introduced into tubes containing 3.0 mL of the Brain Heart Infusion (BHI) medium and incubated for 24 hours at 37°C. After that, the cultures obtained were streaked on plates containing MacConkey agar plus 10 µg/mL cefotaxime and kept in an incubator for 24 hours at 37°C for isolation of cephalosporin-resistant colonies. The isolated colonies were re-picked in the BHI medium and then stored in 10% glycerol at 20°C for preservation (Quinn et al., 1994; 2005).

Biochemical Identification of Bacterial Isolates

The biochemical identification of bacteria belonging to the *Enterobacteriaceae* family was carried out using a set of biochemical tests (Quinn et al., 1994) included in the Enterobacteria Kit (NewProv®, Parana, Brazil), according to the manufacturer's recommendations.

Phenotypic Antibiotic Susceptibility Assays

To determine the bacterial drug resistance profile, the agar diffusion disk method was used, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2013). After incubation of the plate, the diameter of the inhibition halos around each antibiotic disc was measured for subsequent confirmation of sensitivity or resistance. The

following antimicrobials were tested: gentamicin (10 µg), ciprofloxacin (5 µg), sulfatrim (25 µg), ceftazidime (30 µg), amikacin (30 µg), aztreonam (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), tobramycin (10 µg), ceftaxime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), tetracycline (30 µg), amoxicillin (10 µg), amoxicillin + clavulanate (30 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), nalidixic acid (30 µg), cefepime (10 µg), and enrofloxacin (5 µg).

Multiple Antimicrobial Resistance (MAR) Index

To detect possible multidrug resistance, the MAR index was used, defined as a/b, where a was the number of antimicrobials to which the isolates were resistant, and b the number of antimicrobials to which the isolate was exposed. Values above 0.200 indicate samples of high risk for public health (Krumperman, 1983).

The Phenotypic Test for Detection of ESBL-Producing Strains

A double-disc synergy test was performed in which discs containing cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), and aztreonam (30 µg) were placed within 20 mm from a disc containing amoxicillin + clavulanate (20 or 10 µg). Any increase or distortion of the zone of inhibition of one of the antibiotics toward the amoxicillin+clavulanate disk was considered suggestive of ESBL production.

Table 1. Absolute (N) and relative (%) frequency of bacterial isolates belonging to the Enterobacteriaceae family among swabs of the oral and nasal cavity of horses used for animal traction in the municipality of Umuarama, Parana, Brazil, 2016

Bacterial isolates	Isolated Samples			
	Oral Cavity		Nasal Cavity	
	No.	%	No.	%
<i>Escherichia coli</i>	02	33.33	01	16.67
<i>Serratia rubidaea</i>	02	33.33	02	33.33
<i>Citrobacter diversus</i>	01	16,67	0	0
<i>Providenciaal califaciens</i>	0	0	01	16.67
<i>Kluyvera species</i>	01	16.67	0	0
Not identified*	0	0	02	33.33
TOTAL	06	100.00	06	100.00

No. = number of samples, % = Percentage

*No microbes from the Enterobacteriaceae family were identified.

RESULTS

Swabs were collected from 38 horses, 24 (63.16%) males and 14 (36.84%) females from 19 farms. The collected swabs were distributed as follows: 38 from nasal cavities, 38 from oral cavities, and 38 from ear cavities of each animal, totaling 114 samples collected.

Of the 114 samples collected, in 12 (10.23%) it was possible to perform bacterial isolation, six samples from the oral cavity and six from the nasal cavity, and no bacteria were isolated from the ear cavity. Among the samples that grew, eight (72.73%) were obtained from males and two (27.27%) from females.

As for the oral cavity, two (33.33%) strains of *Escherichia coli*, two (33.33%) of *Serratia rubidaea*, one (16.67%) of *Citrobacter diversus* and one (16.67%) of *Kluyvera* species were obtained (Table 1).

From the nasal cavities, two (33.33%) strains of *Serratia rubidaea*, one (16.67%) of *Escherichia coli*, and one (16.67%) of *Providencia alcalifaciens* were obtained, and in two samples (33.33%), identification was not possible because they yielded inconclusive results in the tests (Table 1 and 4).

It was possible to detect bacterial resistance in 12 (100%) isolates, collected from 11 animals and the antibiotics that showed greater resistance were Ertapenem, Cefotaxime, Cefoxitin, Ampicillin, Amoxicillin, Chloramphenicol, and Aztreonam (Table 2).

Regarding the MAR index, 12 (100%) microorganisms were assumed to represent high risk for public health, six (50%) from the oral cavity and six (50%) from the nasal cavity (Table 3).

Regarding the production of ESBL, no sample showed the characteristics indicative of

Table 2. Resistance profiles of bacterial isolates belonging to the Enterobacteriaceae family from nasal and oral cavity swabs of horses used for animal traction in the municipality of Umuarama, Parana, Brazil, 2016

Antibiotics	Bacterial Drug Resistance					
	Nasal Cavity		Oral Cavity		Nasal Cavity + Oral Cavity	
	R	%	R	%	Total	%
Enrofloxacin	0	0	0	0	0	0
Nalidixic Acid	4	66.67	3	50	7	58.33
Ciprofloxacin	0	0	0	0	0	0
Tobramycin	1	16.67	01	16,67	2	16.67
Amikacin	1	16.67	0	0	1	8.33
Gentamicin	1	16.67	0	0	1	8.33
Meropenem	1	16.67	1	16.67	2	16.67
Ertapenem	6	100	6	100	12	100
Imipenem	0	0	0	0	0	0
Ceftazidime	4	66.67	1	16.67	5	41.67
Ceftriaxone	3	50	5	83.33	8	66.67
Cefotaxime	6	100	6	100	12	100
Cefoxitin	6	100	6	100	12	100
Cefepime	1	16.67	1	16.67	2	16.67
Ampicillin	6	100	6	100	12	100
Amoxicillin	6	100	6	100	12	100
Amoxicillin + Clavulanate	2	33.33	0	0	2	16.67
Aztreonam	5	83.33	6	100	11	91.66
Sulfatrim	5	83.33	1	16.67	6	50
Chloramphenicol	6	100	6	100	12	100
Tetracycline	0	0	0	0	0	0

R = resistant, % = Percentage.

Table 3. The MAR index of the isolates belonging to the Enterobacteriaceae family from swabs of the nasal and oral cavity of horses used for animal traction in the municipality of Umuarama, Parana, Brazil, 2016

Samples	MAR Index	
	Oral Cavity	Nasal Cavity
01	0.53	-
02	0.43	0.48
03	0.39	-
04	0.43	-
05	0.48	-
06	0.39	-
07	-	0.48
08	-	0.43
09	-	0.43
10	-	0.81
11	-	0.43

Legend: Krumperman (1983) suggests that values above 0.200 indicate high-risk samples.

Table 4. Profiles of the bacterial isolates belonging to the Enterobacteriaceae family from swabs of the oral and nasal cavity from horses used for animal traction in the municipality of Umuarama, Parana, Brazil, 2016

Sample ID	Bacterial Isolates	
	Oral Cavity	Nasal Cavity
01	<i>Citrobacterdiversus</i>	-
02	<i>Serratia rubidaea</i>	Not identified*
03	-	<i>Providenciaalcalifaciens</i>
04	<i>Kluyveraspecies</i>	-
05	<i>Escherichia coli</i>	-
06	-	<i>Serratia rubidaea</i>
07	-	Not identified*
08	-	<i>Escherichia coli</i>
09	-	<i>Serratia rubidaea</i>
10	<i>Escherichia coli</i>	-
11	<i>Serratia rubidaea</i>	-

Legend: *No microbes from the Enterobacteriaceae family were identified.

ESBL = extended-spectrum β -lactamase.

ESBL production due to the absence of distortion in the inhibition halos.

DISCUSSION

The herd of equines involves several segments, from the distribution of inputs, creation and even the final destination, forming the base of the so-called Agribusiness Complex of the Horse responsible for generating 3.2 million jobs directly and indirectly. This animal species has been used for many years mainly as a means of transport, and in daily tasks of agricultural activities, especially for cattle herd management. Over the years, horses have also spread to other areas of activity, with a strong tendency for leisure, sports, and even therapy (Brasil, 2016).

In all the areas where horses are employed, there is the presence of humans, and it is worth noting that despite the different and many benefits that this animal species offers, it can also pose risks to human health as a result of the transmission of zoonoses and the possibility of transmission of transfer of antibiotic resistance genes between bacteria of animal and human origin, as well as between bacteria of the normal microbiota and pathogenic microorganisms of various origins (Hashimoto et al., 2007; Arias and Carrilho, 2012).

It is known that horses have a commensal microbiota in different regions of the body, e.g. in the oral, nasal, ophthalmic, ear, genital, and anal orifices. Consequently, in this study of the 114 swabs collected from the oral, nasal, and ear cavities, bacterial isolation was possible in 10.23% of the samples, with six (15.79%) samples from the oral cavity and six (15.79%) samples from the nasal cavity. Of these isolates, the bacteria *S. rubidaea* and *E. coli* were the most prevalent, at 33.33% and 25%, respectively.

The detection of *S. rubidaea* in this study does not corroborate other studies on horse species because the other authors detected only the genus *Serratia* sp. as demonstrated by the work of Camara et al. (2013) who detected it in healthy mares and mares with endometriosis from four stud farms in the Municipality of Campos dos Goytacazes (RJ), or *Serratialiquefaciens* was detected in the ophthalmic material of horses of the 1st Cavalry Guard Regiment (CGR) of the Army of Brasilia (DF) in the work by Santana (2015).

S. rubidaea can be found in contaminated water, food, and plants and can survive for years in the environment and be transmitted from animals to humans directly and indirectly (Menezes et al., 2004). This species is responsible for hospital infections and is generally associated with human

infections, particularly affecting the bloodstream or the respiratory or urinary tract, and its pathogenicity may be related to the production of lipase, gelatinase, and DNase enzymes, making them highly resistant to many antimicrobials (Menezes et al., 2004; Tavares, 2015).

Although the identification of *E. coli* has also been reported in the studies by Aguiar et al. (2005) in Botucatu (SP) at 17.30%, by Oliveira et al. (2010) in Agreste and Zona da Mata (PE) at 25.00%, and by Bandeira et al. (2018) on farms in Rio Grande do Sul (RS), at 46.80%, among mares with reproductive problems, mainly endometriosis. Nevertheless, the work by Oliveira et al. (2007) in Agreste and Zona da Mata (PE) detected *E. coli* in vaginal swabs of clinically healthy mares. These results show that *E. coli* exists as a microorganism of the normal flora but may also cause diseases in horses.

The detection of *E. coli* in this study could have been caused by environmental contamination during swab harvesting independent of the site, because this microorganism is generally found in feces and in the environment, and for this reason, can be introduced into the respective sites where the swabs were collected (Oliveira et al., 2007).

Regarding the identification of the other bacterial species, the following species have been detected: *Kluyverasp.*, *C. diversus*, and *P. alcalifaciens* and these species have been detected more frequently in humans (Sarria et al., 2001; Shima et al., 2016; Tellez et al., 2000). These results suggest that these bacterial species are crossing the barriers between farm animals and humans because draft horses have very close contact with their owners or farm workers who use them for traction.

As for the ESBL assays, no sample showed any characteristics indicative of ESBL production, suggesting that up to now in the municipality of Umuarama (PR) there is no evidence of the production of β -lactamases (which is the main mechanism underlying resistance to β -lactam antibiotics, such as penicillin, cephamycins, cephalosporins, monobactams, and carbapenems) among horse species (Silva and Lincopan, 2012). Nevertheless, these results do not corroborate those of Carneiro et al. (2017) who detected indicative strains, which were later confirmed by the gene presence in tracheal and rectal swab

samples of mules from the city of Cachoeira de Macacu (RJ). The use of antimicrobials in animal production has favored the selection of ESBL-producing enterobacteria with a potential for dissemination in the community through direct contact and consumption of contaminated food, which can potentially be established in ecosystems (Silva & Lincopan, 2012).

Regarding bacterial resistance, 12 (100%) samples were found to be resistant to ertapenem, cefotaxime, cefoxitin, ampicillin, amoxicillin, chloramphenicol, and aztreonam. This result probably reflects the incorrect use of antimicrobials in horse species because these drugs are used in cases of nasal septicemia, musculoskeletal, respiratory, cutaneous, ocular, gastrointestinal, and reproductive infections, as well as for surgical prophylaxis (Arias & Carrilho, 2012). Research has shown that the continuous and irrational use of antibiotics exerts selection pressure on the target pathogens and on the microorganisms of the normal microbiota, which affects human, animal, and environmental health, causing profound responses in the environment because these bacteria occur there in complex relationships with other microorganisms, resistance genes, and their hosts (Cooke et al., 2002; Prescott et al., 2002; Guardabassi et al., 2004; Wanderley, 2015).

The results of this work indicate the presence of Enterobacteriaceae strains associated with high bacterial drug resistance in horses used for animal traction in the city of Umuarama (PR) in the State of Parana, Brazil. These findings confirm that these equines can be considered reservoirs of multidrug-resistant microorganisms that can be a serious one health problem considering the possibility of dissemination among bacteria of horses' microbiota, their owners, or farm workers, and even in the environment.

Due to the different functions (agricultural activities, leisure, sports, and even therapy) that this farm animal species can perform, new phenotypic studies followed by molecular assays for the confirmation of resistance genes and ESBL production should be conducted for the purpose of establishing preventive measures and health education aimed at the improvement of public health.

It should be taken into account the proximity that such animals have with their owners, even being considered pets, and their proximity to veterinarians and keepers, and the risks involved in sharing such microorganisms.

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CONFLICT OF INTEREST

All authors confirm that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ETHICS STATEMENT

This project was submitted to the Ethics Committee on Animal Experimentation (CEPEEA) of UNIPAR and was approved under protocol number 30306/2016 on 12/03/2015.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript or the Supplementary Files.

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