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Antimicrobial Resistance Phenotypic Profile of Isolates from Clinical Infections in Dogs*

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ABSTRACT

***Background*:** Antimicrobial resistance is described as a condition in which a micro-organism is able to survive when exposed to an antimicrobial agent. The resistance rates to antimicrobials in companion animals have risen considerably. Studies of local antimicrobial susceptibility profiles are needed as well as education and warning about the use of tests for the identification and susceptibility of pathogenic bacterial strains. The aim of this study was to identify the main antimicrobial resistance in clinical samples of dogs, and to detect multidrug-resistant strains of importance to public health.

***Materials, Methods & Results*:** Bacterial pathogens of 77 dog infections were isolated and their sensitivity profile to antimicrobials was determined. One hundred bacterial isolates were identified. Of these, 61 were Gram-positive (55 *Staphylococcus* spp., 4 *Enterococcus* spp. and 2 *Streptococcus* spp.) and 39 Gram-negative (36 fermenters and 3 non-fermenters). Seventy-nine isolates were considered multiresistant following individual assessment of drugs, and 85 following the evaluation of classes. Only 3 were sensitive to all drugs. Four isolates were resistant to all classes and only sensitive to some antibiotics. Of the 55 samples of Staphylococcus spp., 36 (65.45%) were identified as phenotypically MRS. Two isolates of Enterococcus spp. were resistant to vancomycin (VRE). Also 66.67% (26/39) of the samples were positive for the presumptive test for ESBL. For the MRS-positive isolates detected in this study, chloramphenicol was the antimicrobial that showed superior sensitivity in 74.29% of the cases (27/36); therefore it is considered the most appropriate for treatment of this type of micro-organism. In case of aminoglycosides, when their resistance was checked in MRS isolates, all resistance percentages increased, implying a limited use of this class for such a type of multi-resistant micro-organism. Contrarily, in case of ESBL, a superior sensitivity was observed towards MRS isolates, thus making them a prime treatment choice for the infection caused by these micro-organisms.

***Discussion*:** Literature have reported a gradual increase in multidrug resistance towards antimicrobial agents in veterinary medicine over the past decades. In this study, 64% of multiresistant strains were considered of significant importance, notably MRS (36), VRE (2) and ESBL (26). The early identification of pathogens in animals has become an important step in order to minimize the transmission of antibacterial resistance. The increase in the number of multidrug-resistant bacteria in animals and humans demonstrates the need to develop and implement measures in order to monitor and control the spread of this resistance. It is possible that the increased drug resistance is linked to the constant exposure to these drugs and the subsequent selective pressure, causing the transfer of resistant genes between strains. Carbapenems and glycopeptides should be used with caution in veterinary medicine in order to prevent such processes of selection that develop resistance in micro-organisms to these two classes, which can result in cross-resistance between animals and humans and create obstacles in the treatment of patients, especially for the two drugs mentioned, since they are important for the treatment of nosocomial infections in humans. The resistance percentage towards fluoroquinolones was identified to be higher in Gram-positive isolates, particularly in MRS, which showed 75% resistance against this class (according to the CLSI, resistance to one fluoroquinolone antimicrobial agent provides resistance to other antimicrobials of this class). For ESBL isolates, the resistance was shown to be 50%. The resistance towards the fluoroquinolones and aminoglycosides class can be associated with the expression of the genes that produce ESBL.

Keywords: ESBL, MRS, multidrug resistance, public health, VRE.

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INTRODUCION

Antimicrobial resistance is described as a condition which a micro-organism is able to survive upon exposure to an antimicrobial agent [5]. According to Ishii *et al*. [18], the emergence of multidrug-resistant strains creates failures in the treatment of various infections, and this due to the inappropriate use of antimicrobial drugs, which contributes to the development of bacterial resistance in animals and humans.

Due to the close contact of e.g. dogs and cats to man, and the indiscriminate use of antibiotics in veterinary medicine, these animals become a potential source of resistant bacteria for humans, and vice-versa, potentially leading to the transmission of multiresistant bacteria interspecies [17].

Several steps must be taken to combat multidrug-resistant micro-organisms in order to prevent the severe impact on public health, especially the conscious use of antimicrobials, a correct administration route and a monitoring of the resistance profile by antibiogram techniques [15].

The resistance rates towards antimicrobials in companion animals have risen considerably. Studies of local antimicrobial susceptibility profiles are needed as well as proper education and warning about the use of tests for the identification and susceptibility of pathogenic bacterial strains [18,27]. Thus, the aim of this study was to identify the main antimicrobial resistance in clinical samples of dogs and to detect the multidrug-resistant bacterial pathogens involved.

MATERIALS AND METHODS

Samples of 77 dogs, treated at the Veterinary Hospital of the State University of Maringá (UEM), Regional Campus Umuarama (CAU), were used in this study. The samples were sent to the Animal Microbiology Laboratory - UEM / CAU.

The samples were initially incubated in Brain Heart Infusion broth - BHI (Brain Heart Infusion)[1] at 36°C for 2 to 18 h, then plated on Blood agar (5% sheep blood defibrillated in Nutrient Agar[1] and MacConkey Agar[1]. The isolates were identified based on colony morphology and biochemical reaction [1].

Antimicrobial susceptibility tests were performed through the disk diffusion method on Muller Hinton agar[1] according to Bauer *et al*. [6]. The zone sizes were interpreted by CLSI [7,8]. The antimicrobial agents tested were β-lactam penicillins: penicillin G (10U); β-lactam aminopenicillin: amoxicillin (10 μg) and ampicillin (10 μg); β-lactam/β-lactamase inhibitors combinations: amoxacillin-clavulanic acid (30 mcg) and ampicillin-sulbactan (20 μg); β-lactam penicillinase-stable penicillins: oxacillin (1 μg); β-lactam cephalosporin: first generation - cephalexin (30 mcg) and cephalothin (30 μg), 3rd generation - ceftriaxone (30 μg), ceftazidime (30 μg) and cefotaxime (30 μg) and 4th generation - cefepima (30 μg); β-lactam cephems: cefoxitin (30 mcg); β-lactam monobactams: aztreonam (30 μg); β-lactam carbapenems: imipenem (10 mcg) e meropenem (10 μg); Glycopeptides: vancomycin (30 μg); Polypeptides: polymyxin (300 μg); Aminoglycosides: gentamicin (10 μg), streptomycin (10 μg), amikacin (30 μg), neomycin (30 μg) and tobramycin (10 μg); Macrolides: 14-membered rings - erythromycin (15 μg) and 15-membered rings - azithromycin (15 μg); Lincosamides: clindamycin (2 μg); Ansamycin: rifampin (5 μg); Phenicols: chloranphenicol (30 μg); Nitrofurantoin: nitrofurantoin (10 mcg); Fluoroquinolone: enrofloxacin (05 μg), norfloxacin (10) μg, ciprofloxacin (5 μg) and levofloxacin (5 μg); Tetracyclines: tetracycline (30 μg) and doxycycline (30 μg); Folate pathway inhibitors: trimethoprim-sulfamethoxazole (25 μg) [Newprov®][2].

Phenotypic detection of multidrug-resistant strains that are of public health significance was performed by disk diffusion with: oxacillin and cefoxitin to Methicillin-Resistant *Staphylococcus* (MRS) [7,8]; synergism between amoxicillin-clavulonic acid and aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefepime to Extended-spectrum beta-lactamase (ESBL) producing Gram-negative [8,30]; and vancomycin to Vancomycin-Resistant *Enterococcus* (VRE) [7].

The Multiple Antibiotic Resistance index (MAR) was calculated as the number of resistant ratings across the total number tested [19]. The Multiple Antimicrobial Classes Resistance index (MCR) was calculated as the ratio between the number of classes considered resistant (at least one drug per class) and the total number of classes tested. A ratio larger than or equal to 0.25 was considered multirresistant [23].

The results were submitted for descriptive analysis in order to calculate absolute and relative frequencies [27].

RESULTS

The 77 analyzed samples originated from different locations/distribution systems. The bacterial

frequency in the different samples is shown in Table 1. The overall resistance rate was 45.24% for skin infections, 35.13% for conjunctivitis, 33,12% for cystitis, 29.92% for piometras, 28.82% for otitis, 22.62% for superior respiratory tract infections and 37.22% for other types of infections.

Pure colonies were isolated from 59 samples, whereas mixed cultures with two bacterial types were isolated in 14 samples, with three bacterial types in 3 samples and with four bacterial types in just one sample. 100 bacterial isolates were identified, as shown in Table 2. The antimicrobial resistance rates (MAR), for which indices ≥ 0.2 indicate multidrug resistance, varied between 0 and 0.93. In this study, 79 out of 100 isolates (79%) showed multidrug resistance, and nine isolates showed a MAR index higher than 0.8 (8 *Staphylococcus* CP and *Enterobacter* sp.). Only three isolates were sensitive to all drugs tested. Considering the MCAR index, 85% of the samples had an index greater than 0.25, and showed resistance to three or more classes of antimicrobials. The average MCAR index of the samples studied was 0.49. Four samples were resistant to at least one antimicrobial of all classes tested (two *Staphylococcus* CP, one *E. coli* and one *Enterobacter* sp.).

A total of 2723 tests with antimicrobial drugs were performed in this study: 35.92% (978) were considered resistant, and 9.51% (259) were considered to have intermediate resistance, and finally 45.43% (1237) of the samples showed only slight resistance level. Samples reported with intermediate resistance were counted as resistant for statistical purposes, since it is not advisable to use these antimicrobials in clinical medicine. The resistance rates of 100 isolates for each antimicrobial are shown in Figure 1.

Oxacillin and cefoxitin are, according to CLSI [7], predictive drugs for resistance in *Staphylococcus* spp. to all beta-lactam antibiotics, which is called MRS. In this study, of the 55 samples of *Staphylococcus* spp., 36 (65.45%) were resistant to oxacillin and/or cefoxitin, which demonstrates the presence of MRS.

The results of this study, through use of the disk diffusion technique, show that of the 55 samples of *Staphylococcus* spp., 32 (58.18%) were susceptible in vitro to vancomycin and 23 need further assessment for minimal inhibitory concentration (MIC) for this antimicrobial. According to CLSI [7], vancomycin resistant *Staphylococcus* (VRS) can only be reported, through MIC. For *Enterococcus* spp., through disk diffusion technique, which were found two VRE strains, both with MAR rates higher than 0.2.

In this study, 66.67% (26/39) of Gram-negative isolates tested positive in the presumptive test for detection of ESBL. Of these, seven (26.92%) *E. coli* were isolated, as were four (15.38%) *Providencia* spp. and *Proteus* spp., three (11.54%) *Enterobacter* spp., and two (7.69%) *Pantoea agglomerans*, *Citrobacter* spp., *Pseudomonas* spp. and *Serratia* spp. Of the 26 ESBL samples, only three (11.54%) showed a MAR index below 0.2. This confirms the presence of multidrug resistance in these micro-organisms.

The resistance profiles of the main multiresistant micro-organisms (MRS, VRE and ESBL) are shown in Figures 2 and 3; the indices MAR and MCAR are shown in Table 3.

Table 1. Distribution in overall frequency and MAR frequency ≥0.2 of the bacterial strains found in different organ systems of infected dogs from Veterinary Hospital of the State University of Maringa, PR, Brazil.

Bacterial isolates	Gram positive		Gram negative				Total	
Sample	Total	MAR ≥0.2	Fermenters		No Fermenters		Total	MAR ≥0.2
			Total	MAR ≥0.2	Total	MAR ≥0.2		
Skin	17	16	12	10	0	0	29	26
Ophthalmic	15	10	6	4	0	0	21	16
Ear infections	13	7	1	1	2	2	16	13
Vaginal	8	6	9	6	0	0	17	13
Urinary	1	0	4	3	1	1	6	4
nasal discharge	4	1	2	1	0	0	6	2
Mammary	1	1	0	0	0	0	1	1
Bone	0	0	1	1	0	0	1	1
Oral	1	1	0	0	0	0	1	1
Others	1	1	1	1	0	0	2	2
Total	61	43	36	27	3	3	100	79

MAR = Multiple Antibiotic Resistance index.

Table 2. Distribution frequency, MAR indices and MCAR medium and frequency of multidrug-resistant bacteria found in bacterial isolates from dog infections from Veterinary Hospital of the State University of Maringa, PR, Brazil.

Bacterial isolates		Frequency	Average MAR	Freq MAR ≥0.2	Average MCR	Freq MCR ≥0.25
Cocci G+	*Staphylococcus* spp.	55	0.447	41	0.566	45
	Enterococcus spp.	4	0.333	2	0.432	3
	Streptococcus spp.	2	0.206	1	0.285	1
Total G+		61	0.329	44	0.428	49
Gram -	Fermenters					
	Escherichia coli	12	0.433	10	0.552	10
	Enterobacter spp.	4	0.506	4	0.597	4
	Proteus spp.	4	0.416	4	0.520	4
	Providencia spp.	4	0.282	3	0.440	4
	Serratia spp.	3	0.487	3	0.630	3
	Pantoea agllomerans	3	0.448	2	0.533	2
	Citrobacter spp.	2	0.517	2	0.625	2
	Salmonella spp.	1	0.226	1	0.380	1
	Unidentified	3	0.452	3	0.537	3
	No Fermenters					
	Pseudomonas spp.	3	0.639	3	0.797	3
Total G-		39	0.441	35	0.561	36
Grand Total		100	0.385	79	0.494	85

*MAR = Multiple Antibiotic Resistance index; MCR = Multiple Antimicrobial Classes Resistance index.

Table 3. Distribution frequency, MAR indices and MCAR from MRS isolated, VRE and ESBL isolated from dog infections in Veterinary Hospital of the State University of Maringa, PR, Brazil.

	Total	Total (%)	Frequency MAR >2	Average MAR	Frequency MCR	Average MCR
MRS	36 (55)	65.45	35	0.575	35	0.63
ESBL	26 (39)	66.67	23	0.448	25	0.46
VRE	2 (4)	50	2	0.532	2	0.6

*MAR = Multiple Antibiotic Resistance index; MCR = Multiple Antimicrobial Classes Resistance index; MRS = Methicillin-Resistant *Staphylococcus*; ESBL = Extended-spectrum beta-lactamase; VRE = Vancomycin-Resistant *Enterococcus*.

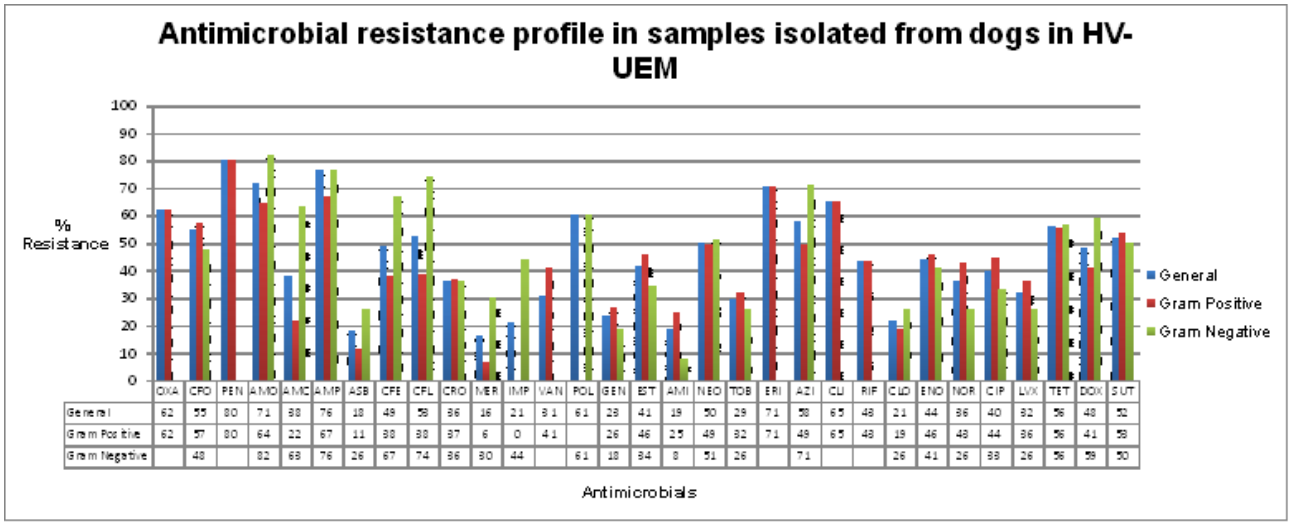
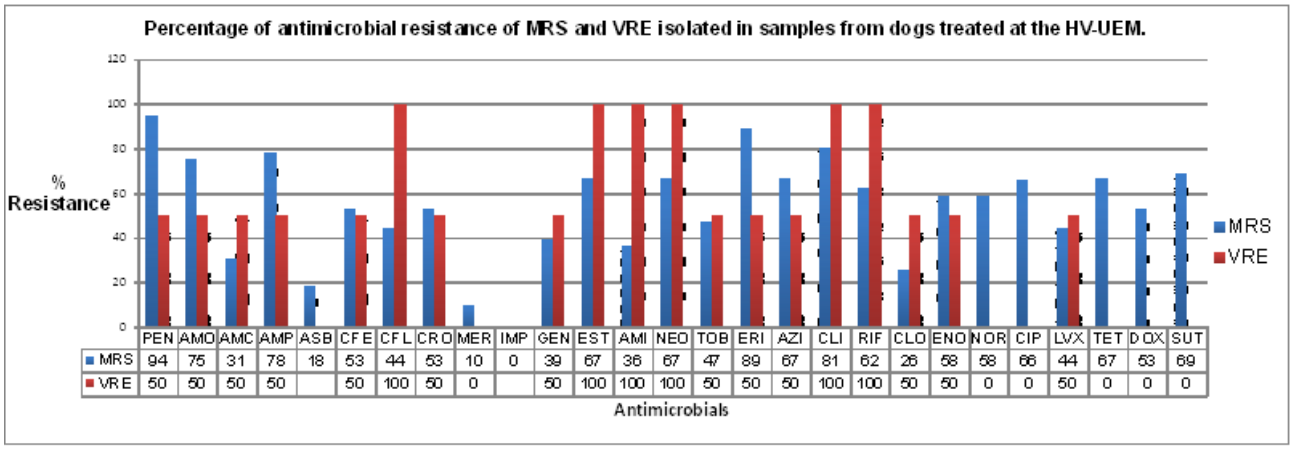
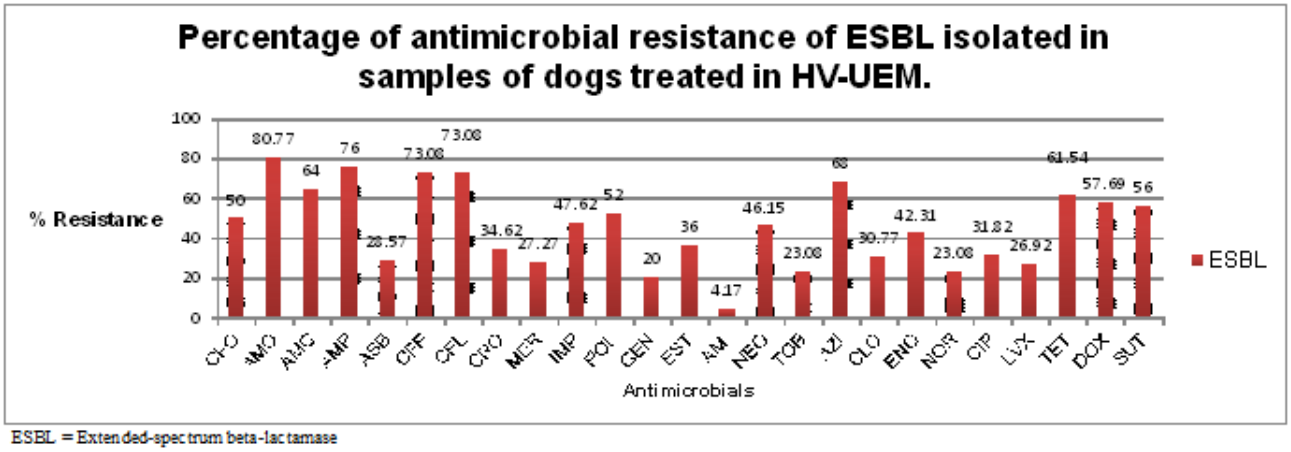


Figure 1. Antimicrobial resistance profile in samples isolated from dogs in HV-UEM, PR, Brazil.



MRS = Methicillin-Resistant *Staphylococcus*; VRE = Vancomycin-Resistant *Enterococcus*

Figure 2. Percentage of antimicrobial resistance of MRS and VRE isolated in samples from dogs treated at the HV-UEM, PR, Brazil.



ESBL = Extended-spectrum beta-lactamase

Figure 3. Percentage of antimicrobial resistance of ESBL isolated in samples of dogs treated in HV-UEM, PR, Brazil.

DISCUSSION

With regard to the multidrug resistance index, Arias *et al*. [3] studied samples of infected surgical wounds, and found 19 out of 23 samples (82.6%) to have a MAR index ≥ 0.2, three of which with a value of 1. Sfaciotte *et al*. [27] isolated 89.4% of the analyzed samples with a MAR index ≥ 0.2. Both studies thus registered a high index medium, however only a few samples were studied and only a few antimicrobial drugs were tested. Some authors have reported a gradual increase in multidrug resistance to antimicrobials in veterinary medicine over the past decades [2,22].

In this study, in 64% (64/100) of tested samples, multiresistant strains of high importance were detected, such as MRS (36), VRE (2) and ESBL (26). The early identification in animals has become an important step in minimizing the transmission of antibacterial resistance. The increase in the number of multidrug-resistant bacteria in animals and humans demonstrates the need to develop and implement measures that monitor and control the diffusion of this resistance [18,28]. It is possible that the increased resistance is linked to the constant exposure to these drugs and the subsequent selective pressure caused by the transfer of resistance genes between strains [29].

Methicillin resistance is the most important mechanism of antimicrobial resistance in *Staphylococcus* sp. identified so far. Resistant isolates of *S. pseudintermedius* methicillin (MRSP) have been reported worldwide in veterinary clinics and hospitals. MRSP bacteria are recognized as a major pathogen due to

multidrug resistance and the associated difficulty in treatment of infections [17,28].

One of the most important mechanisms of resistance found in the Enterobacteriaceae family of micro-organisms is the desactivation through hydrolysis of the beta-lactam ring by enzymes. These bacteria are beta-lactamasis producers of extended spectrum (ESBL), which gives resistance to antimicrobial agents of the cephalosporins and monabactams classes [25], although they do not hydrolyze cephamycins and carbapenems. These antimicrobials are also desactivated by beta-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) [20]. A major study on ESBL detection on small animals was conducted in Germany and resistance genes were detected for *E. coli*, *Salmonella enterica*, *Proteus mirabilis* and *Enterobacter cloaceae* through isolation from wounds, as well as urinary and respiratory infections [13].

Of 19 methicillin-sensitive strains of *Staphylococcus* sp. (MSS), 84.21% were sensitive to cephalexin, 78.95% to cephalothin and 89.47% to ceftriaxone. Therefore, their use is advisable. These antimicrobials are recommended to be used in a medical and surgical veterinary clinic environment. There is however no such recommendation for MRS samples.

The carbapenems are beta-lactam antimicrobial agents, and they are usually the last choice for treatments. They are used for ESBL producing micro-organisms and nowadays are often used for the treatment of human nosocomial infections. However, the presence of resistant isolates for carbapenems is already being reported through the expression of carbapenemases [31].

In this study, 30.3% and 43.75% of ESBL samples showed some level of resistance to meropenem and imipenem respectively. It indicates that there is a requirement for the development of more specific tests to detect the presence of resistance genes that encode carbapenemases. Although there are no clear rules that prohibit the use of carbapenems in veterinary medicine, these antibiotics should be used with caution in order to avoid the pressure of selection of resistant clones and the transmission of resistance to other bacteria, potentially affecting humans [28].

Vancomycin is a major antimicrobial used for the treatment of infections caused by *Enterococcus* spp. and MRS in human medicine. However, with the emergence of VRE and VRSA, there are only a few therapeutic agents capable of treating infections caused by these micro-organisms [16]. This agrees with the results found in this study, where the two identified VRE strains were resistant to most of the antimicrobial classes tested. In veterinary medicine, no samples of VSRA were identified [22].

Under these circumstances, the presence of VRE, VRSA and MRS should be extensively monitored in veterinary hospital environments, reporting the ocurrence of any cases and investigating its origin. Carbapenems and glycopeptides also should be used with caution in veterinary medicine in order to prevent the selection of resistant micro-organisms towards these two classes. Otherwise, cross-resistance between animals and humans can occur, which can vastly complicate the treatment of patients in these two areas.

With regard to the aminoglycoside class of antibiotics, five drugs were tested and all of them showed a better efficacy in Gram-negative isolates, except for neomycin. When the resistance in MRS isolates was checked, all percentages showed an increase. It limits the use of this class for such types of multi-resistant micro-organisms. The opposite proved to be the case for ESBL, showing better sensitivity for these drugs, and as a result making them eligible for treatment of infections caused by these micro-organisms.

Macrolides, lincosamides and streptogramin B form the MLSB group of antibiotics because, despite different formulas, they have the same action mechanism and epidemiologically the cross-resistances between these three classes are very important [14] due to their wide use in veterinary medicine. In this study, 94.4% (34/36) of the MRS strains were resistant to at least one antibiotic of the MLSB group.

Fluoroquinolones, especially enrofloxacin, are one of the main antibiotics used in veterinary medicine, because as well as their easy acquisition they prove efficient for most empirical antimicrobial treatments, and present relative safety, a broad action spectrum and strong diffusion in key organs and tissues [24].

The percentage resistant to fluoroquinolones was higher in Gram-positive isolates, particularly in MRS, which showed 75% of resistance against the class (according to the CLSI, resistance to antimicrobial agent of fluoroquinolones confers resistance to the entire class). For ESBL isolates, the resistance was 50%. The resistance class of fluoroquinolones and aminoglycosides may be associated with the expression of gene producers of ESBL [10].

For isolated MRS detected in this study, chloramphenicol was the antimicrobial that showed the highest sensitivity, 74.29% (27/36); thus it is the most suitable antimicrobial for treatment of this type of micro-organism. In the PABA class of inhibitors, Sfaciotte *et al*. [27] found resistance in 100% of the samples studied, Arias *et al*. [3] concluded 92.3% (12/13) in samples of contaminated and infected wounds, and according to Dal-Bo *et al*. [12] 75% to *Staphylococcus* sp. and 50% to Gram-negative bacilli. These high rates of antimicrobial resistance of the sulfa class can be explained due to its wide use since many years in veterinary medicine, often without adequate criteria [11].

CONCLUSION

The results of this study indicate that the main isolated micro-organism infection in different canine organ systems is *Staphylococcus* spp., followed by Gram-negative bacilli fermenting sugars, which belong to Enterobactereacea family.

Multiresistant strains of high importance to public health were detected, such as MRS, VRE, ESBL. It demonstrates the need for constant monitoring of bacterial resistance profiles, which vary over the years and differ from site to site. The use of tests for bacterial identification and their susceptibility to antimicrobials can help in the appropriate selection of an antimicrobial agent. It is essential for medical and surgical environments due to the high bacterial resistance rates seen in this and other studies, as well as the monitoring of local resistances with the continued use of certain antimicrobial drugs.

MANUFACTURERS

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***Ethical approval*.** All procedures illustrated were undertaken under a project licence approved by the Committee of Ethical Conduct in the use of Animals in Experimentation, State University of Maringá, with reference number 064/14.

***Declaration of interest*.** The authors declare that there are no conflict of interests regarding the publication of this paper.

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