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Original Research Paper

Multidrug-Resistant Bacterial Pathogens Assessment in Canine Ophthalmic Infections

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Abstract: The objective of this study was to identify the main microorganisms associated with ophthalmic infections and determine the resistance profile of these isolates against antimicrobial drugs. 26 bacterial isolates from 18 canine ophthalmic infections were submited to the phenotypic resistance profile for 36 drugs of 12 classes of antimicrobials, research of multidrug-resistant strains with importance in public health and detection of *Staphylococcus mec*A gene by PCR. The bacterial isolates were identified as *Staphylococcus* spp. (n = 18), *Enterococcus* spp. (n = 1), enterobacteria (n = 6) and *Pseudomonas* spp. (n = 1). The percentage of resistance and intermediate resistance were 42.48% (n = 325). Considering separate antimicrobials drugs, 18 isolates were characterized by multidrug resistant, while by the assesment of resistance to class, 20 isolates were multiresistant. In the phenotypic detection, 61.11% (11/18) of *Staphylococcus* spp. were predicted by Methicillin-Resistant *Staphyloccus* (MRS), whereas the genotypic detection, 38.89% (7/18) were carriers of the *mec*A gene. Two enterobacterias were considered producers of expectro Extended of Betalactamase (ESBL). EUCAST was more reliable for detecting MRS strains than the CLSI. The present study detected multiresistant isolates of great importance and are involved in cases of public health, such as MRS, MRSMLSb, ESBL, very important to be readily identified and controled so as to prevent the spread of this type of resistance.

Keywords: Multiresistant, MRS, *mec*A, ESBL, Public Health

Introduction

Superficial tissues, such as skin and mucosa, are colonized by different agentes because they are in constant contact with the environment. In addition to the frequent exposure, the ocular surface is rich in nutrients, which makes a favorable environment for the colonization of microorganisms, ranging throughout life (Prado *et al*., 2005). These microorganisms, of ocular microbiota, act as an important defense mechanism (Wang *et al*., 2008), preventing the emergence of pathogens by competing for nutrients, secreting antimicrobial substances and to stimulate the local immune response (Moeller *et al*., 2005). Although not considered pathogenic, when a break occurs the barrier protection of the ocular surface, a decrease of immunity, as well as stress or another factor that initiates an imbalance between host and agent, these microorganisms can seep into the corneal stroma or injure the conjunctiva and initiate an infectious process (Solari *et al*., 2004).

The amount of resident bacterial population in the conjunctiva is small, especially being found Grampositive bacteria of the *Staphylococcus* and *Streptococcus* (Prado *et al*., 2005), genus as well as Gram-negative

bacteria, but these when in large numbers, may indicate changes in eye health (Spinelli *et al*., 2010).

There are several studies that have identified the resident microbiota of the conjunctiva and all showed the prevalence of Gram-positive, even in different animal species, such as dogs (Anvisa, 2013), horses, capybara, capuchin monkey, domestic ferret (Montiani-Ferreira *et al*., 2006; 2008a; 2008b) or even aquatic habitat animals as beavers, showed the same profile (Cullen, 2003).

The main genus of microorganisms isolated in ophthalmic changes animals are *Staphylococcus* (Prado *et al*., 2005); Wang *et al*., 2008), followed by *Streptococcus*, *Pseudomonas* and *Escherichia* coli (Tolar *et al*., 2006) In dogs, *Staphylococcus pseudintermedius* is identified as the main agente (Montiani-Ferreira *et al*., 2006).

In ophthalmology, the use of antimicrobials is carried out so much to prevention and for the treatment of diseases, therefore, it is extremely important to determine the susceptibility of microorganisms against antimicrobial agents in external ocular diseases because the indiscriminate use of these agents in minor infections affect the treatment of more serious diseases. The most recommended antibiotics in ophthalmic practice are gentamicin, tobramycin, neomycin, chloramphenicol and ciprofloxacin, mainly in *Staphylococcus* (Varges *et al*., 2009).

The objective of this study was to identify the main microrganisms associated with dogs ophthalmic infections and determine the resistance profile of these isolates against antimicrobial drugs.

Material and Methods

There were assessed 26 bacterial strains of 18 ophthalmic infections in dogs. The samples were collected from animals at the Clinic Medical for Small Animals of The Veterinary Hospital of State University of Maringa, Brazil by sterile swabs. The samples were initially incubated in Brain Heart Infusion broth – BHI (OXOID®) at 36°C for 2 to 18 h, then plated on Blood agar (5% sheep blood defibrillated in Nutrient Agar-OXOID®) and MacConkey agar (OXOID®), incubated at 36ºC for 24/48 h. The isolates were identified based on colony morphology and biochemical reaction (Anvisa, 2013).

Antimicrobial susceptibility tests were performed by disk diffusion method on Muller Hinton agar (OXOID®) according to Bauer *et al*. (1966) and the zone sizes were interpreted by Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines and by European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013). 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 ampicillinsulbactan (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®).

Phenotypic detection of multidrug-resistant strains of public health significance was performed by disk diffusion with: Oxacillin and cefoxitin to Methicillin-Resistant *Staphylococcus* (MRS) (CLSI, 2013; EUCAST, 2013); erythromycin and clindamycin to Macrolide-Lincosamide-Streptogramin B (MLSb) of *Staphylococcus* (Kim *et al*., 2004); synergism between amoxicillin-clavulonic acid and aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefepime to Extended-Spectrum Beta-Lactamase (ESBL) producing Gram-negative (Souza Junior *et al*., 2004); and vancomycin to Vancomycin-Resistant *Enterococcus* (VRE) (CLSI, 2013). The Multiple Antibiotic Resistance index (MAR) was calculated by the number of resistant ratings over the total tested, ≥0.2 values were considered multirresistant, according Krumperman (1983). The Multiple Antimicrobial Classes Resistance index (MCR) was calculated by the ratio between the number of classes considered resistant (at least one drug per class) and the total number of classes tested, ≥0.25 values were considered multirresistant, according Ngoi and Thong (2013).

The DNA extraction of staphylococci strains was performed by Doyle and Doyle (1987). The PCR was performed according to Sfaciotte *et al*. (2015a) with primers SMAswF (5'- GAT GAT ACC TTC GTT CCA C-3' nt 622-640) and SMAswR (5'GTA TGT GCG ATT GTA TTG C-3' nt 917-935) that amplify a 314 bp.

This study was accepted by the Ethics Committee of the Federal University of Paraná, Palotina sector (CEUA/Palotina) with the number of Protocol No. 04/2014. The results were submitted to descriptive analysis to calculate the absolute and relative frequencies.

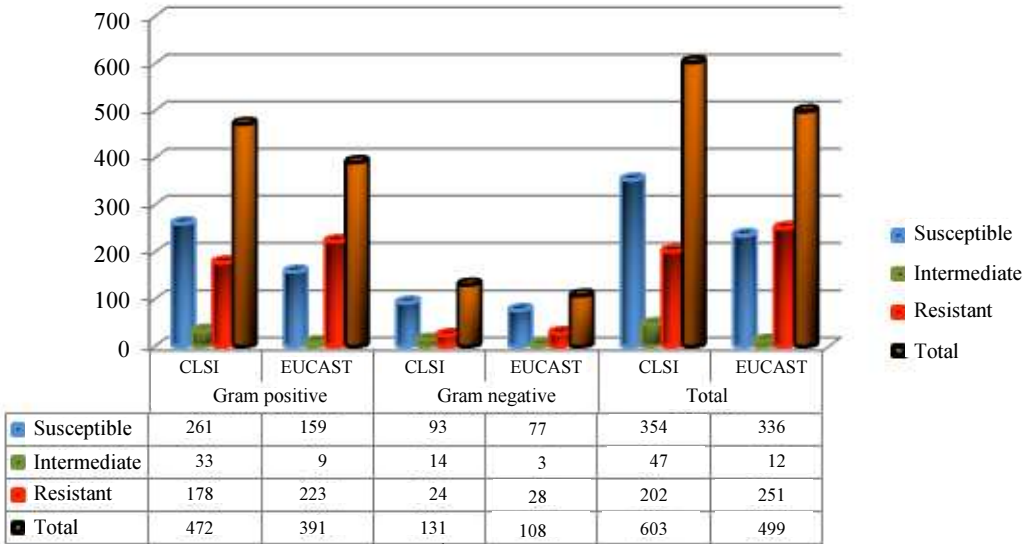
Results

The microorganisms isolated from external ophthalmic infections in dogs and their multiple resistance levels are described in Table 1.

A comparative interpretation of antimicrobial susceptibility between CLSI and EUCAST have been done, according to each bacterial type (Fig. 1), as well as average MAR (Fig. 2).

Phenotypic detection MRS showed that 47.05% (8/17) of isolates of *Staphylococcus* spp. were resistant to oxacillin and 52.94% (9/17) to cefoxitin by the interpretation of CLSI, while of EUCAST interpretation, 76.47% (13/17) showed resistance to cefoxitin (based on interpretation of *Staphylococcus pseudointermedius*). The oxacillin resistance by EUCAST must be performed by MIC not evaluated in this study. Compared to the detection of the *mec*A gene by PCR, two positive samples for *mec*A were susceptible to cefoxitin and oxacillin for CLSI interpretation while all 7 PCR positive were resistant to cefoxitin for EUCAST interpretation (Table 2).

Regarding the resistance profile found in bacterial strains studied, just three drugs had percentages above 70% of resistance (penicillin, R = 84.2%; ampicillin, R = 76%; clindamycin, R = 80.77%).



	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST
	Gram positive		Gram negative		Total	
Susceptible	261	159	93	77	354	336
Intermediate	33	9	14	3	47	12
Resistant	178	223	24	28	202	251
Total	472	391	131	108	603	499

Fig. 1: Comparative interpretation of antimicrobial susceptibility tests parameters of bacterial pathogens of canine external ophthalmic infections

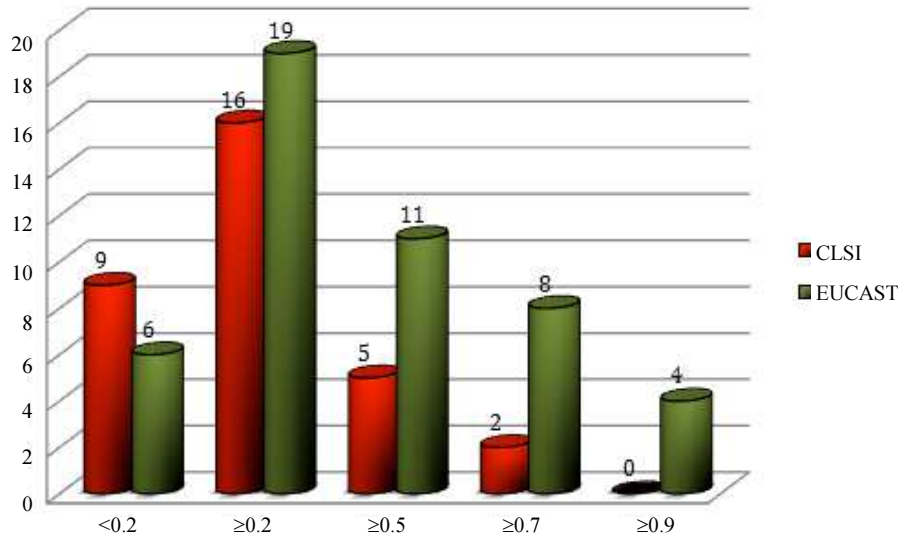


Fig. 2: Comparative interpretations of Multiple Antibiotic Resistance index (MAR) with different parameters of bacterial pathogens of canine external ophthalmic infections

Table 1: Distribution in frequency, percentage and multidrug resistance by CLSI and EUCAST of bacterial pathogens of canine external ophthalmic infections

	Bacterial strains	Frequency (n)	Percent (%)	MAR[1]	MAR[2]
Gram +	*Staphylococcus*	17	68	0.38	0.58
	Enterococcus	1	4	0.15	0.37
	Total	18	72	0.37	0.57
Gram -	Enterobacteria				
	Escherichia coli	2	8	0.35	0.38
	Pantoea	2	8	0.15	0.32
	Salmonella spp.	1	4	0.10	0.13
	Enterobacter	1	4	0.10	0.12
	Total	06	24	0.20	0.28
	Non-fermenting				
	Pseudomonas	1	4	0.00	0.00
	Total	01	4	0.00	0.00
Total		25	100	0.31	0,48

MAR: Multiple Antibiotic Resistance index; [1]CLSI; [2]EUCAST

Table 2: Results of phenotypic and genotypic assessments carried out in *Staphylococcus* spp. isolated from canine external ophthalmic infections

		Oxacillin		Cefoxitin		
Interpretation	PCR	R	S	R	S	Total
CLSI	Positive	5	2	5	2	7
	Negative	3	7	4	6	10
	Total	8	9	9	8	17
EUCAST	Positive	-	-	7	0	7
	Negative	-	-	6	4	10
	Total	-	-	13	4	17

PCR: Polimerase chain reaction for mecA detection

Table 3: Percentage of bacterial resistance to antimicrobial agents of canine external ophthalmic infections

	CLSI			EUCAST		
	G+	G-	Total	G+	G-	Total
PEN	77,78	-	77,78	76,47		76,47
AMO/AMP	76,47	-	76,47	76,47	50	69,56
AMC	0	16,67	4,35	-	33,33	33,33
APS	29,41	0	21,74	-	0	0
CFL	29,41	66,67	39,13	76,47	-	76,47
CRO	23,53	28,57	25	76,47	33,33	65,22
MER	5,88	14,29	8,33	76,47	14,29	58,33
GEN	33,33	14,29	28	38,89	14,29	32
AMI	5,88	0	4,17	17,65	0	12,5
TOB	23,53	14,29	20,83	41,18	28,57	37,5
ERI	55,56	-	55,56	52,94	-	52,94
CLI	64,71	-	64,71	64,71	-	64,71
RIF	44,44	-	44,44	70,59	-	0,59
CLO	16,67	0	12,5	17,65	0	13,04
NOR	50	14,29	40	50	50	50
CIP	66,67	57,14	64	61,11	50	58,33
LEV	33,33	28,57	32	33,33	28,57	32
TET	77,78	33,33	66,67	76,47	-	76,47
DOX	33,33	33,33	33,33	0	-	0
SUT	64,71	50	60,87	72,22	50	66,67

G+: Gram Positive; G-: Gram Negative; PEN: Penicillin; AMO/AMP: Amoxicillin/ampicillin; AMC: Amoxacillin-clavulanic acid; APS: Ampicillin-sulbactan; CFL: Cephalothin; CRO: Ceftriaxone; MER: Meropenem; GEN: Gentamicin; AMI: Amikacin; TOB: Tobramycin; ERI: Erythromycin; CLI: Clindamycin; RIF: Rifampin; CLO: Chloranphenicol; NOR: Norfloxacin; CIP: Ciprofloxacin; LEV: Levofloxacin; TET: Tetracycline; DOX: Doxycycline; SUT: Trimethoprim-sulfamethoxazole

Drugs considered less resistant were ceftriaxone (R = 26.92%), chloramphenicol (R = 19,23%), amikacin (R = 11.54%), ampicillinsulbactam (R = 5%), amoxicillin-clavulonate (R = 15.38%), imipenem (R = 0%) and meropenem (R = 0%); whereas samples reported with intermediate resistance were computed as resistant for statistical purposes, once it is not advisable its use in clinical veterinary medicine. Resistance percentages front of antibacterial agents of the general samples are in the Table 3.

Discussion

This study showed similar results to those found by Oria *et al*. (2013) to the identification of bacterial types involved in ophthalmic infections which were 64.51% of the samples identified as being Gram-positive and 35.48% Gram-negative. When compared to the identification of the bacterial genus, the present study found similar numbers to Zacarias Junior *et al*. (2012) for *Staphylococcus* (66%), but higher than Oria *et al*. (2013) with found 38%, however, similar numbers for *E. coli* and *Enterobacter* spp., 27.27% and 18.18% respectively. As Santos *et al*. (2009) 100% of cultured samples showed at least one kind of bacterial growth.

The predominance of Gram-positive isolates is because staphylococci is part of the resident flora of the mucosa and skin, so when there is an imbalance between the agent and the host, these microorganisms can become pathogenic (Prado *et al*., 2005; Wang *et al*., 2008). As to Gram-negative bacteria, particularly enterobacteria, are considered opportunistic agents in the majority of infections, thus, the isolation of this bacterial type in this study, particularly bacteria that are not commonly associated with ocular infections, such as *Salmonella* spp. and *Pantoea agglomerans*, can be suggested by environmental contamination and/or poor hygiene conditions.

Researches reports a gradual increase in multidrug resistance to antimicrobials in veterinary medicine (Mota *et al*., 2005; Arias and Carrilho, 2012), a fact proven in this study, where 69.23% (18/26) had an index MAR ≥0.2. With the increase in the number of drugs tested, this index tends to have lower values, but with greater reliability, as occurred in this study that evaluated an average of 36 antimicrobials by samples, being tested at least one antimicrobial of 12 drug classes.

When it comes to external ophthalmic infections, the main antimicrobials used in veterinary practice are the aminoglycosides (tobramycin and gentamicina), chloramphenicol and, in some cases, tetracycline (Bedford and Jones, 2001). This study showed good susceptibility to tobramycin (74.08%), of which only five (all *Staphylococcus*) proved to be resistant to this antibiotic, which was not evidenced by Subtil (2010), who found high rates of resistance, but these values similar to Zacarias Junior *et al*. (2012) who observed 78.26% susceptibility. Gentamicin also presents a low resistance, 30.77% (8/26), of which seven (87.5%) were *Staphylococcus* spp. and one *Pseudomonas* spp., according to literature and slightly higher compared with Zacarias Junior *et al*. (2012) who found a resistance of just 19.56%, where no Gram-negative sample showed resistance to this antimicrobial.

The samples have low resistance to chloramphenicol, 19.23% (5/26), where only one Gram-negative sample, *Pseudomonas* spp., was resistant and four *Staphylococcus* (all phenotypically identified as MRS). This good susceptibility goes according to Subtil (2010).

When tested tetracycline, more than half of the isolates were resistant, 69.23% (18/26) of which 72.2% (13/18) of resistance found in *Staphylococcus*, beyond resistance of *Enterococcus* spp. and *Pseudomonas* spp., values similar to those reported by Subtil (2010) in Portugal and lower than those found by Zacarias Junior *et al*. (2012) 80.43%. Of the samples phenotypically identified as MRS, 90.91% (10/11) were resistant to tetracycline and when identified the *mec*A gene, all were resistant to tetracycline.

The oxacillin is a semi synthetic drug of the beta-lactam class and, according to the CLSI (2013), is the drug for predicting resistance to all beta-lactam antibiotics in *Staphylococcus pseudintermedius* also associated with resistance to cefoxitin. When a sample shows phenotypic resistance to oxacillin and cefoxitin, indicates the presence of the *mec*A gene providing lower binding affinity of β-lactam ring (Kim *et al*., 2012; Cartwright *et al*., 2013). In this study, of the 18 samples of *Staphylococcus* spp. 38.89% (7/18) were positive for detection of *mec*A gene in the PCR reaction, a value higher than found by Pereira *et al*. (2009), 15%.

MRS isolates were associated to multiple resistance to another antibiotic addition to resistance to beta-lactam class (Table 4 and 5). Resistance to fluoroquinolones is relatively common and in this study, of the 11 MRS identified phenotypically, nine (81.81%) were resistant to at least one antibiotic of the class, results similar to Asbell *et al*. (2008) while in samples which the *mec*A gene was detected, only one sample were sensitive to all the antimicrobial agents.

Antimicrobial classes of macrolides, lincosamides and streptogramin B have the same antimicrobial resistance mechanism, inhibiting the protein synthesis, forming MLSb group (Fiebelkorn *et al*., 2003). 100% of samples which the *mec*A gene were considered MLSb resistant, being that Kim *et al*. (2004) also found a 97% resistance to at least one antibiotic of group MSLb in MRSA.

After the discovery of multiresistant Gram-positive bacteria, especially MRS, antimicrobial class of glycopeptides, vancomycin and teicoplanin, has been, for many years, the only alternative for the treatment against these micro-organisms in medicine. Of the 18 samples of *Staphylococcus*, 13 (72.23%) were susceptible to vancomycin in disk diffusion test, in five (27.77%), the MIC test for correct assessment is required. Until now has not reported any sample VISA or VRSA in veterinary medicine due to scarce amount of study front of resistance to glycopeptides (Monchique, 2013; Sfaciotte *et al*., 2015b).

In the present study were detected two samples ESBL, a strain of *Pantoea agglomerans* (MAR = 0.44) and other *Pseudomonas* spp. (MAR = 0.5) and the two samples were sensitive to the carbapenems tested. According to Zacarias Junior *et al*. (2012) there is a deficiency in susceptibility studies on antimicrobial isolates of microorganisms Gram-negative of external ophthalmic diseases in dogs.

Table 4: Phenotypic, genotypic and multidrug resistance index in *Staphylococcus* spp. isolated from canine external ophthalmic infections, considering CLSI parameters

Bacterial isolate	Oxacilin	Cefoxitin	PCR	MAR	MCR
ST 01	R	S	-	0.56	0.58
ST 02	R	R	+	0.79	0.83
ST 03	R	R	+	0.75	0.83
ST 04	R	R	-	0.45	0.67
ST 05	R	R	+	0.50	0.83
ST 06	R	R	-	0.63	0.67
ST 07	R	R	+	0.63	0.67
ST 08	R	R	+	0.33	0.50
ST 09	R	R	-	0.37	0.33
ST 10	R	R	+	0.43	0.50
ST 11	R	S	-	0.37	0.58
ST 12	S	S	-	0.00	0.00
ST 13	S	S	-	0.14	0.25
ST 14	S	S	-	0.23	0.33
ST 15	S	S	-	0.10	0.17
ST 16	S	-	-	0.39	0.42
ST 17	S	-	-	0.04	0.08
ST18	S	S	+	0.13	0.17
Total	11	9	7	0.38	0.45

PCR: Polimerase chain reaction for mecA detection; MAR: Multiple Antibiotic Resistance index; MCR: Multiple antibiotic Class Resistance

Table 5: Antibiotic resistance of MRS and MSS isolated from canine external ophthalmic infections, considering CLSI parameters

Antibiotic	MRS (n = 7)	MSS (n = 11)
Penicillin	7 (100%)	7 (63.64%)
Oxacillin	6 (85.7%)	5 (45.45%)
Cefoxitin	6 (85.7%)	3 (27.27%)
Vancomycin	3 (42.86%)	2 (18.18%)
Streptomycin	3 (42.86%)	4 (36.36%)
Gentamicin	3 (42.86%)	4 (36.36%)
Amikacin	0 (0%)	3 (27.27%)
Neomycin	3 (42.86%)	5 (45.45%)
Tobramycin	3 (42.86%)	3 (27.27%)
Erythromycin	6 (85.7%)	5 (45.45%)
Azithromycin	5 (71.43%)	4 (36.36%)
Clindamycin	6 (85.7%)	8 (72.73%)
Rifampin	4 (57.14%)	4 (36.36%)
Chloramphenicol	2 (28.57%)	2 (18.18%)
Enrofloxacin	6 (85.7%)	3 (27.27%)
Norfloxacin	5 (71.43%)	3 (27.27%)
Ciprofloxacin	5 (71.43%)	4 (36.36%)
Levofloxacin	4 (57.14%)	2 (18.18%)
Tetracycline	7 (100%)	6 (54.54%)
Doxycycline	4 (57.14%)	3 (27.27%)
Trimethoprimsulfamethoxazole	6 (85.7%)	6 (54.54%)
MAR	0.51	0.30
MCR	0.62	0.37

MRA: Methicillin Resistant *Staphylococcus*; MSS: Methicillin Susceptible *Staphylococcus*; MAR: Multiple Antibiotic Resistance index; MCR: Multiple antibiotic Class Resistance

Regarding the *Enterococcus* isolated, the sample was sensitive to the disk diffusion test with vancomycin. With the emergence of *Enterococcus* resistant to Vancomycin (VRE), this group of microorganism has become one of the most important clinically resistant bacteria throughout the world, because there are few therapeutic agents capable of treating infections caused by this group. However, due to the low number of isolates of *Enterococcus* and failure to detect VRE strains in the present study, we can not make an assessment of the background about this multiresistant microorganism.

Comparing the interpretation of antimicrobial resistance ratings by CLSI and EUCAST is clear a greater resistance found according to EUCAST. EUCAST was more reliable for detecting MRS strains, however, there is also an increase in false positives.

The bacterial resistance profile varies over the years and differs from region to region, so its monitoring should be constant and should not be ignored by veterinary professionals, both clinical and surgeons. The prudent choice of the adopted antimicrobial therapy reduces the use of antibiotics and consequently the development of bacterial resistance by the selection, particularly in hospital settings.

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Author's Contributions

Ricardo Antonio Pilegi Sfaciotte: Conducted the experiment, summarized the date.

Lincoln Garcia Coronel, Alessandra Snak, Jéssica Tainá Bordin and Vanessa Kelly Capoia Vignoto: Contributed the execution of the study.

Leandro Kiyoshi Yamamoto: Contributes to the collection of samples.

Sílvia Cristina Osaki: Contributed to the planning and execution of the study and the laboratory analysis.

Sheila Rezler Wosiacki: Conceptualized and supervised the research, drafted the manuscript and ran statistical tests. All authors have read and approved the manuscript.

Ethics

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

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