Original Research Paper

Antimicrobial Resistance in Bacterial Pathogens of Canine Otitis

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Abstract: Otitis is one of the most frequent infections in dogs. This is attributed to the misuse of drugs enabling generation of multi-resistant micro-organisms. The emergence of multiresistant bacterial strains in veterinary medicine is a reality that must be studied and evaluated by the professionals. The objective of this study was to isolate and evaluate the antimicrobial susceptibility of bacterial pathogens of otitis in dogs. Otologic swabs were collected from 36 dogs with clinical otitis. 41 bacterial strains were isolated and antimicrobial susceptibility tests were performed by disk diffusion method with 34 antimicrobial agents. Presence of the resistance gene *mecA* of *Staphylococcus* was examined for 22 strains of staphylococci by PCR. A total of 1108 ratings antimicrobial agents were performed. The percentage of drug resistance was 34.66% (n = 384) of the assessments with partial or total resistance. Major bacterial pathogens were *Staphylococcus* spp. (65.85%), *Pseudomonas* spp. (12.19%) and Enterobacteria species (19.51%). 53.66% of the isolates were considered multiresistant. Antimicrobial agents considered most resistant in the strains studied were penicillin (75.00%), tetracyclin (50.00%), amoxicillin (48.78%), trimethoprim-sulfamethoxazole (46.15%), clindamycin and rifampicin (43.24%). 11 strains were phenotypically characterized as MRS, 4 genotypically as MRS, 2 as MLSB-MRS and 2 as gram negative ESBL-producing.

Keywords: Otitis, Canine, Pathogens, Antimicrobial Resistance, *Staphylococcus*

Introduction

Otitis is one of the most frequent infections in dogs. It is a disease of multifactorial causes and can manifest acutely or chronically. Predisposing factors such as anatomical conformations of both the ear canal and the ears, changes in microflora and immune suppression are some of the main observed.

The normal ear canal microflora is varied consisting of coagulase-positive and coagulase-negative *Staphylococcus* spp., β-haemolytic *Streptococcus* spp., *Proteus* spp., Escherichia coli and Pseudomonas spp. (Hariharan *et al*., 2006; Petrov *et al*., 2013). Changes to this microflora can lead to the development of opportunistic and also pathogenic bacteria (Oliveira *et al*., 2012).

According to (Oliveira *et al*., 2012; Oliveira *et al*., 2005; 2006b) main bacterial pathogens of canine otitis are *Staphylococcus* and *Pseudomonas* species. Other bacterial species found in smaller percentages are *Streptococcus*, *Escherichia coli*, *Proteus* and *Enterococcus* species (Hariharan *et al*., 2006).

Frequently observed failure of treatment of ear infections. This is attributed to the misuse of drugs enabling generation of multi-resistant micro-organisms, that result in reduced efficacy of the drugs. This occurrence has increased concomitantly with routine use of antibiotics, especially broad-spectrum drugs that are able to act on a large number of bacterial species. Not performing tests for agent identification and

susceptibility favors the generation of resistant strains, making it essential to carry out these (Ishii *et al.*, 2011).

The emergence of multiresistant bacterial strains in veterinary medicine is a reality that must be studied and evaluated by the professionals.

The objective of this study was to isolate and evaluate the antimicrobial susceptibility of bacterial pathogens of otitis in dogs.

Materials and Methods

Sample Collection

Otologic swabs were collected from 36 dogs with clinical otitis in the Veterinary Hospital of State of Maringá, Brazil between March, 2012 and September, 2014. The samples were initially incubated in Brain Heart Infusion broth - BHI (OXOID®) at 36°C for 2 to 18 hours, then plated on Blood agar (5% sheep blood defibrillated in Nutrient Agar-OXOID®) and MacConkey agar (OXOID®), incubated at 36°C for 24/48 h. The isolates were identified based on colony morphology and biochemical reaction.

Antimicrobial Resistance

Antimicrobial susceptibility tests were performed by disk diffusion method on Muller Hinton agar (OXOID®) according to (Bauer *et al.*, 1966) as recommended by the Clinical and Laboratory Standards Institute (CLSI) and the zone sizes were interpreted per CLSI guidelines (CLSI, 2008; 2013). The following 13 classes with 34 antimicrobial agents were tested: β-lactam penicillins: penicillin G (10U); β-lactam aminopenicillin: amoxicillin (10 μg) e ampicillin (10 μg); β-lactam/β-lactamase inhibitors combinations: amoxacillin-clavulanic acid (30 μg) and ampicillin-sulbactan (20 μg); β-lactam penicillinase-stable penicillins: oxacillin (1 μg) (just in gram positive); β-lactam cephems cephalosporin: first generation-cephalexin (30 μg) and cephalothin (30 μg) and 3rd generation - ceftriaxone (30 μg); β-lactam cephems cephamycin: cefoxitin (30 μg); β-lactam carbapenems: imipenem (10 μg) e meropenem (10 μg); Glycopeptides: vancomycin (30 μg) (just in gram positive); Polypeptides: polymyxin (300 μg) (just in gram negative); Aminoglycosides: gentamycin (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); Oxazolidinone: linezolid (10 μg) (just in gram positive); Phenicols: chloranphenicol (30 μg); Nitrofurantoin: nitrofurantoin (10 μg); Fluoroquinolone: enrofloxacin (5 μg), norfloxacin (10 μg), ciprofloxacin (5 μg) and levofloxacin (5 μg); Tetracyclines: tetracycline (30 μg) and doxycycline (30 μg); Folate pathway inhibitors: sulfisoxazole (300 μg) and sulfamethoxazole-trimethoprim (25 μg) (NEWPROV®).

Phenotypic detection of multidrug-resistant strains of public health significance was performed by disk diffusion with: oxacillin to Methicillin-Resistant *Staphylococcus* spp. (MRS) (CLSI, 2008); erythromycin and clindamycin to Macrolide-Lincosamide-Streptogramin B (MLS(B)) of *Staphylococcus* spp. (Kim *et al.*, 2004); synergism between amoxicillin-clavulonic acid and aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefepime to Extended-Spectrum β-Lactamase (ESBL)-producing gram negative (Sousa Junior *et al.*, 2004).

The Multiple Antibiotic Resistance (MAR) index was calculated by the number of resistant ratings over the total tested according (Krumperman, 1983).

Staphylococcus spp. mecA Detection

Presence of the resistance gene *mecA* of *Staphylococcus* spp. was examined for 22 strains of staphylococci by PCR. The DNA extraction was performed by (Doyle *et al.*, 1987). 200 μL of Tris-EDTA (TE) was added in to microtubes with isolated colonies and centrifuged at 8000×G for 5 min. The supernatant was discarded and the precipitation was suspended in 600 μL of CTAB (Cetyl Trimethyl Ammonium Bromide) and 40 μL of CIA (Chloroform/isoamyl alcohol), heated in a water bath at 65°C for 30 min. Over 800 μL of CIA was added and centrifuged at 12000×G for 5 min. 600 μL of the supernatant was transferred to another microtube and was added 600 μL of cold isopropanol. The mixture was incubated at -20°C for 16 h and centrifuged at 13,500×g for 20 min at 4°C. The supernatant was discarded and the DNA was dried in laminar flow. The purified DNA was eluted in 200 μL TE.

The PCR was performed 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, designed by Gene Runner software (version 3.0-Copyright © 1994 Hasting Software, Inc.). In the PCR 2μL of extracted DNA was added to 0.4 pmol of each primers, 0,5 mM each dNTP (Invitrogen Inc., Carlsbad, CA, USA), 1.5 unit of *Taq* DNA Polymerase (Invitrogen), 1x PCR buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 3.0mM $MgCl_2$ and ultrapure sterile water to a final volume of 25 μL. The amplification consisted of the following time and temperature conditions: one step of 10 min/95°C followed by 30 cycles at 30 sec/95°C, 30 sec/52°C and 1 min/72°C and a final extension step of 10 min/72°C. Amplicons were analyzed by electrophoresis in a 1% agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA) stained with 0.5 μg/mL ethydium bromide and visualized under UV light.

Statistical Analysis

The results were submitted to descriptive analysis to calculate the absolute and relative frequencies.

Results

The simple bacterial growth was found in 31 samples, in 13.88% (5/36) two bacterial strains were identified, totaling 41 bacterial strains isolated (Table 1).

A total of 1108 ratings antimicrobial agents were performed. The percentage of drug resistance was 28.70% (n = 318) and intermediate resistant, 5.95% (n =

66) totaling 34.66% (n = 384) of the assessments with partial or total resistance (Fig. 1).

The resistance to antimicrobial agents is contained in Table 2 and Fig. 2.

About 11 strains phenotypically characterized as MRS, 2 as MLSB-MRS and 2 as gram negative ESBL-producing (*Proteus* spp. and *Pseudomonas* spp.) were detected.

The MAR index medium was 0.293. Of the 41 strains evaluated, 53.66% (22/41) showed ≥0.2 MAR and were considered multiresistant by (Krumperman, 1983) (Table 1).

Table 1. Frequency and percentage distribution and evaluation of Multiple Antibiotic Resistance (MAR) index found in bacterial pathogens of canine otitis

| Bacterial strains		n	MAR %	n (médio)	n MAR ≥0.2	n MAR ≥0.5	MAR ≥0.75
G+	*Staphylococcus* spp.	27	65,85	0.234	12	4	1
	Unidentified bacillus	1	2,44	0.09	0	0	0
	Total	28	68,29	0.229	12	4	1
G -	Enterobacteria						
	Halfnia alveii	2	4,88	0.340	1	1	0
	Proteus spp.	1	2,44	0.440	1	0	0
	Providencia spp.	1	2,44	0.518	1	1	0
	Serratia spp.	1	2,44	0.120	0	0	0
	Klebsiella spp.	1	2,44	0.160	0	0	0
	Salmonella spp.	1	2,44	0.448	1	0	0
	Total	8	19,51	0.401	5	3	1
	Non-fermenting						
	Pseudomonas spp.	5	12,19	0.478	5	2	0
	Total	5	12,19	0.478	5	2	0
TOTAL		41	100,00	0.293	22	9	2
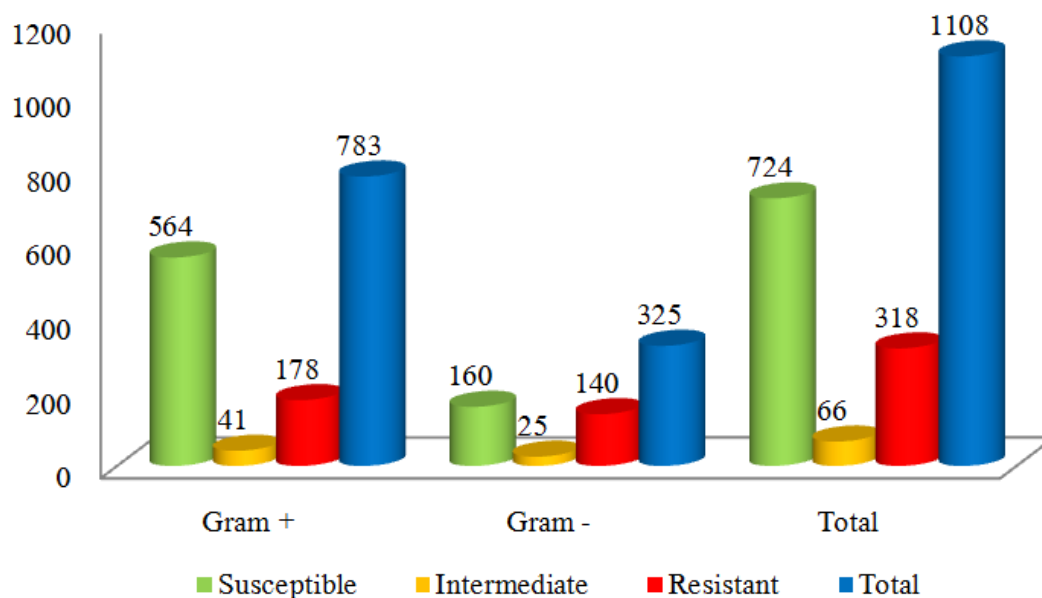
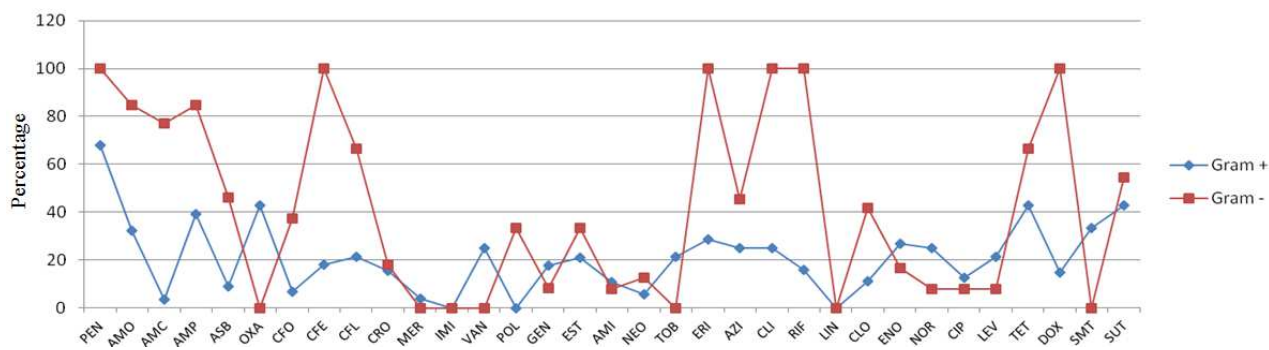Fig. 1. Susceptibility tests of bacterial pathogens of canine otitis

Fig. 2. Percentage of resistance to antimicrobial agents found in bacterial pathogens of canine otitis

Table 2. Frequency distribution and percentage of antimicrobial resistance found between bacterial strains obtained from canine otitis

Antimicrobial agents	Gram positive			Gram negative			Total		
	R	n	%	R	n	%	R	n	%
Penicillin	19	28	67.86	8	8	100	27	36	75.00
Amoxacillin	9	28	32.14	11	13	84.62	20	41	48.78
Amoxacillin-clavulanic acid	1	28	3.57	10	13	76.92	11	41	26.83
Ampicillin	11	28	39.29	11	13	84.62	22	41	53.66
Ampicillin-sulbactan	2	22	9.09	6	13	46.15	8	35	22.86
Oxacillin	12	28	42.86	0	0	0.00	12	28	42.86
Cefoxitin	1	15	6.67	3	8	37.50	4	23	17.39
Cephalexin	4	22	18.18	6	6	100.00	10	28	35.71
Cephalothin	6	28	21.43	8	12	66.67	14	40	35.00
Ceftriaxone	4	26	15.38	2	11	18.18	6	37	16.22
Meropenem	1	25	4.00	0	13	0.00	1	38	2.63
Imipenem	0	21	0.00	0	13	0.00	0	34	0.00
Vancomycin	7	28	25.00	0	0	0.00	7	28	25.00
Polymyxin B	0	1	0.00	4	12	33.33	4	13	30.77
Gentamycin	5	28	17.86	1	12	8.33	6	40	15.00
Streptomycin	4	19	21.05	4	12	33.33	8	31	25.81
Amikacin	3	28	10.71	1	13	7.69	4	41	9.76
Neomycin	1	18	5.56	1	8	12.50	2	26	7.69
Tobramycin	6	28	21.43	0	13	0.00	6	41	14.63
Erythromycin	8	28	28.57	5	5	100.00	13	33	39.39
Azithromycin	7	28	25.00	5	11	45.45	12	39	30.77
Clindamycin	7	28	25.00	9	9	100.00	16	37	43.24
Rifampin	4	25	16.00	12	12	100.00	16	37	43.24
Linezolid	0	3	0.00	0	0	0.00	0	3	0.00
Chloranphenicol	3	27	11.11	5	12	41.67	8	39	20.51
Enrofloxacin	7	26	26.92	2	12	16.67	9	38	23.68
Norfloxacin	7	28	25.00	1	13	7.69	8	41	19.51
Ciprofloxacin	3	24	12.50	1	13	7.69	4	37	10.81
Levofloxacin	6	28	21.43	1	13	7.69	7	41	17.07
Tetracycline	12	28	42.86	8	12	66.67	20	40	50.00
Doxycycline	4	27	14.81	9	9	100.00	13	36	36.11
Sulfametrin	2	6	33.33	0	0	0.00	2	6	33.33
sulfamethoxazole-trimethoprim	12	28	42.86	6	11	54.55	18	39	46.15
TOTAL	178	783	22.73	140	325	43.08	318	1108	28.70

About 4 of 22 *Staphylococcus* spp. strains were positive for *mecA* detection by PCR, genotypically characterized as MRS. Only one of these was resistant to oxacillin by disk diffusion, the others three were susceptible.

Discussion

The main micro-organisms found in this study were *Staphylococcus* spp. and *Pseudomonas* spp., with frequency of 65.85% and 12.19 respectively. In 1990, (Oliveira *et al.*, 2006a) also found similar etiology, being isolated 61.02% of *Staphylococcus* spp., 12.71% of *Pseudomonas* spp., still having 14.41% of *Streptococcus* spp. and other agents with low prevalence. (Oliveira *et al.*, 2005) isolated 53.04% of *Staphylococcus* spp. and 25.05% of *Pseudomonas* spp. (Oliveira *et al.*, 2006a) isolated 55% of *Staphylococcus* spp. and 10% of *Pseudomonas* spp. (Oliveira *et al.*, 2012) identified 46.75% of

Staphylococcus spp. and 8.8% of *Pseudomonas* spp. Therefore it is observed that bacterial etiology of dog's ear canal infections is very similar, not depending on geographic region nor conditions such as animal race, age, or sex.

In this study antimicrobial drugs considered more resistant on isolated bacterial were penicillin (R=75.00%), ampicillin (R=53.66%), tetracycline (R=50.00%), amoxacillin (R=48.78%) and sulfamethoxazole-trimethoprim (R=46.15%).

Since 1990's, etiology and susceptibility of antimicrobials are studied in cases of canine otitis (Megid *et al.*, 1990) found high sensitivity to gentamycin, ranging from 91.7 to 100% depending on the pathogen; amikacin with 90.6% of sensitivity to *Staphylococcus* spp.; erythromycin, ranging from 82% to *Staphyloccocus* and 100% to *Streptoccocus;* chloramphenicol with sensitivity of 100% to *Streptococcus* and *Proteus*, 82% to *Staphylococcus* and 30% to *Pseudomonas*; tetracyclin with resistance ranging from 63.5 to 12.5% depending on the pathogen; and sulfonamid, with sensitivity of 22.2 to 25.7%

Oliveira *et al.* (2005) found great susceptibility studying Coagulase-Positive *Staphylococcus* (CoPS) and negative (CoNS) and *Pseudomonas* as well. Joining the groups CoPS and CoNS, the most sensitive drug considered were: 100% cefoxitin; ≥90% amoxacillin + clavulonic acid, imipenem and netilmycin; ≥80% cefotaxin, ciprofloxacin and enrofloxacin; ≥70% tobramycin, cephalexin, gentamycin, chloramphenicol and amikacin; ranging from 70 to 20% oxacillin, neomycin, tetracycline and ampicillin, ≤40% penicillin. To *Pseudomonas* spp. the most sensitive drugs were ciprofloxacin (97.1%), imipenem (90.1%, tobramycin (85.9%) and enrofloxacin (73.3%) while the least sensitive were β-lactam penicillins and cephalosporins, aminoglycosides (neomycin 19.2%, gentamycin 53.3% and amikacin 59.7%) and chloramphenicol (5.4%).

Oliveira *et al.* (2006a) studying the resistance of *Staphylococcus intermedius* detected the greatest resistance to erythromycin (27.78%), penicillin G (25.96%), tetracycline (24.07%), ampicillin (16.67%) and sulfamethoxazole (11.11%). Drugs considered totally sensitive were amikacin, kanamycin, gentamycin, neomycin, tobramycin, imipenem, cephalothin, cephalexin, defoxitin and ticarcillin.

Oliveira *et al.* (2012) detected resistance of 2.66% to norfloxacin, 12.75% to gentamycin, 13.94% to ofloxacin, 19.78% to tobramycin, 12.63% to ciprofloxacin, 27.63% to cephalexin and 30.84% to neomycin.

Data obtained in this study corroborate with most of other studies performed with the profile and etiology of resistance to antimicrobials, being found a low prevalence of resistance in all of them to most of antimicrobials drugs to *Staphylococcus* and greater

resistance to *Pseudomonas* justified by the high intrinsic resistance of this microorganism. 13 antimicrobial classes were tested in this study, totalizing 34 antimicrobial agents of tested.

β-lactam penicillins drugs (penicillin R=75.00%) and aminopenicillin (ampicillin R= 53.66% and amoxacillin R=48.78%) showed high level of resistance, however when associated with β-lactamase inhibitors (sulbactan with ampicillin R=22.86% and clavulonic acid with amoxacillin R=26.83%) it was noted the improvement in sensitivity to drugs in 57.41% and 45% of the cases respectively. So this study recommends the association between aminopenicillin with β-lactamase inhibitors.

The semi synthetic oxacillin β-lactam drug is, according to CLSI (2008), a drug for pre-editting of resistance in *Staphylococcus* spp. to all β-lactam, associated to resistance to cefoxitin. According to Kim *et al.* (2012) and Cartwright *et al.* (2013) resistance to oxacillin and cefoxitin shows phenotypically the presence of *mecA* gene. On the other hand resistance to cefoxitin with sensitivity to oxacillin, shows the presence of *mecC* gene, which is responsible for the production of a protein that links additional penicillin (PBP2a) that provides low affinity of linking to β-lactam drugs. *Staphylococcus* spp. that carries this gene is called MRS. Several studies show the existence of MRS in Medicine Veterinary.

In this study 11 (40.74%) of 27 *staphylococci* strains were resistant to oxacillin. However, only 4 carried the *mecA* gene of which 3 were oxacillin-susceptible. Nevertheless, all four will be reported as MRS, making 14.81% (4/27). These results show that the phenotypic detection of oxacillin-resistance in not totally related to the presence of *mecA* gene as well as there are possible other genes of resistance to β-lactam agents that not only the *mecA*, gene being so important as this.

Tested β-lactam cephalosporins agents showed low resistance level, ranging from 35.00% to cephalothin, 35.71% cephalexin and 16.22% to ceftriaxone. However they showed great resistance in gram negatives (cephalexin and cephalothin).

Carbapenems have a chemical structure similar to penicillin, but with chemical characteristics which give them greater affinity to PBPs, showing greater potency and an expanded antibacterial spectrum. Resistance to carbapenems is already considered a problem to public health in many countries (Ribeiro, 2013). Carbapenems evaluated in this study have shown 2.63% of resistance to meropenem (only in gram positive). Although there are no rules that forbid the use of carbapenems in Veterinary Medicine, these antimicrobials must be used very carefully in order to avoid pressure in selecting resistant clones and resistance transmission to other bacteria, potentially some contact with humans.

After the discovery of multiresistant gram positive bacteria, specially the MRS, antimicrobial class of glycopeptides vancomycin and teicoplamin have been

the last alternative for the treatment against these micro-organisms for many years in Medicine (WHO, 2009). In Veterinary Medicine the resistance to glycopeptides has been hardly studied due to little use. According to Haenni *et al*. (2010) and Monchique (2013), VISA strains (Vancomycin Intermediate Resistance *Staphylococcus aureus*) and VRS (Vancomycin-Resistant *Staphylococcus aureus*) have not been reported in Veterinary Medicine yet. The results of this study, using disk-diffusion show that in 27 samples of *Staphylococcus* spp. 21 were susceptible *in vitro* to vancomycin and 7 need new evaluation through antimicrobial test by Minimum Inhibitory Concentration (MIC).

Aminoglycoside class showed one of the best level of susceptibility in this study, ranging from 7.69% to 25.81%, as well as phenicols.

Macrolides, Lincosamides and Streptogramin B form MLSB group of antibiotics, although having different formulas, they present the same mechanism action, inhibiting the protein synthesis through the link to the 23S receptor of rRNA that takes part of the 50S sub-unity of the bacterial ribosome. Since 1956, soon after the introduction of erythromycin in the market, *Staphylococcus aureus* resistance to MLSb group (Leclercq, 2002) has already been seen. In Medicine Veterinary, clindamycin is largely used, also indicated to infections caused by *Staphylococcus,* mainly the MRS (Fiebelkorn *et al*., 2013). However, Kim *et al*. (2004) while analysing the presence of resistance to MLSb group in *Staphylococcus aureus* found out that 97% of MRSA (Meticillin-Resistant *Staphylococcus aureus*) showed resistance to at least one of the antibiotics of this group. Epidemiologically, the crossed-resistance among these 3 classes of antimicrobials is very important Dipersio and Dipersio (2005) once they are largely used in Veterinary Medicine taking to the increase of resistance of animal origin.

The tested macrolides showed resistance of 39.39% to erythromycin and 30.77% to azithromycin. Resistance to clindamycin was found in 43.24% of the studied samples. 11.11% (03/27) of *Staphylococcus* spp. resistant to MLSb group were detected with the two tested drugs, two of them being characterized as MRS.

The class of fluoroquinolones, according to CLSI (2008) must be reported together, where resistance to a drug indicates the resistance of the whole class. Percentages of resistance of 10.81% to cyprofloxacin, 19.51 % to norfloxacin, 23.68% to enrofloxacin and 17.07% to levofloxacin were found, showing greater significant resistance in gram positives, being these drugs of great value for the empirical treatment by gram negative bacteria.

Conclusion

Major bacterial pathogens were *Staphylococcus* spp. (65.85%), *Pseudomonas* spp. (12.19%) and Enterobacterial species (19.51%). 53.66% of these isolates were considered multiresistant. The percentage of drug resistance was 34.66% (n = 384) of the assessments with partial or total resistance. Antimicrobial agents considered most resistant in the strains studied were penicillin (75.00%), tetracyclin (50.00%), amoxicillin (48.78%), trimethoprim-sulfamethoxazole (46.15%), clindamycin and rifampin (43.24%). 11 strains were phenotypically characterized as MRS, 4 genotypically as MRS, 2 as MLSB-MRS and 2 as gram negative ESBL-producing. All these strains are considered of importance in public health.

Acknowledgment

Funding Information

Author's Contributions

R.A.P. Sfaciotte: Conducted the experiment, summarized the date.

J.T. Bordin and V.K.C. Vignoto: contributed the the execution of the study.

P.M. Munhoz: Reviewed statistical analysis procedures.

A.A. Pinto, M.J.B. Barbosa and R.M. Cardozo: Contibuted to the experiment analysis and manuscript preparation.

S.C. Osaki: Contributed to the planning and execution of the study and the laboratory analysis.

S.R. 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.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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