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 submitted 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 meCA 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 assessment of resistance to class, 20 isolates were multiresistant. In the phenotypic detection, 61.11% (11/18) of Staphylococcus spp. were predicted by Methicillin-Resistant Staphylococcus (MRS), whereas the genotypic detection, 38.89% (7/18) were carriers of the meCA gene. Two enterobacteria were considered producers of espectro 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, meCA, 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 agent (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 microorganisms 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 36ºC 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: Amoxicillin-clavulanic acid (30 mcg) and ampicillin/subactan (20 µg); β-lactam penicillinase-stable penicillins: Oxacillin (1 µg); β-lactam cephalosporin: First generation - cephalaxin (30 mcg) and cephalothin (30 µg), 3rd generation - ceftriaxone (30 µg), cefazidime (30 µg) and cefotaxime (30 µg) and 4th generation -cefepima (30 µg); β-lactam cepham: 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: Rifampicin (5 µg); Phenicolis: Chloramphenicol (30 µg); Nitrofurantoin: Nitrofurantoin (10 mcg); Fluoroquinolones: Enrofloxacin (05 µg), norfloxacin (10 µg), ciprofloxacin (5 µg) and levofloxacín (5 µg); Tetracyclines: Tetracycline (30 µg) and doxycycline (30 µg); β-lactam 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); erthyromycin and clindamycin to Macrolide-Lincosamide-Streptogramin B (MLSb) of *Staphylococcus* (Kim et al., 2004); synergism between amoxicillin-clavulonic acid and aztreonam, ceftazidime, cefotaxime, ceftriaxone, cepefime 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 multiresistant, 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 multiresistant, 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 GCC 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 mecA gene by PCR, two positive samples for mecA 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, \( R = 80.77\% \)).

![Fig. 1: Comparative interpretation of antimicrobial susceptibility tests parameters of bacterial pathogens of canine external ophthalmic infections](image1)

![Fig. 2: Comparative interpretations of Multiple Antibiotic Resistance index (MAR) with different parameters of bacterial pathogens of canine external ophthalmic infections](image2)
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\textsuperscript{1} | MAR\textsuperscript{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**         | 6            | 24          | 0.20                    | 0.28                    |
| Non-fermenting    |              |             |                         |                         |
| Pseudomonas       | 1            | 4           | 0.00                    | 0.00                    |
| **Total**         | 04           | 4           | 0.00                    | 0.00                    |
| **Total**         | 25           | 100         | 0.31                    | 0.48                    |

MAR: Multiple Antibiotic Resistance index; \textsuperscript{1}CLSI; \textsuperscript{2}EUCAST

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

| Interpretation | Oxacillin | Cefoxitin |
|----------------|-----------|-----------|
|                | 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| 69.56 - 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: Amoxicillin-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%), ampicillin-sulbactam (R = 5%), amoxicillin-clavulanate (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 mecA 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 mecA 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 mecA 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 mecA 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 mecA gene were considered MLSb resistant, being that Kim et al. (2004) also found a 97% resistance to at least one antibiotic of group MSld 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 | Oxacillin | 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|
| ST 18             | 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       | 7 (100%)    | 2 (18.18%)   |
| Tetracycline       | 7 (100%)    | 6 (54.54%)   |
| Doxycycline        | 4 (57.14%)  | 3 (27.27%)   |
| Trimethoprim/sulfamethoxazole | 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.

**Acknowledgement**

We thanks the staff of the Veterinary Hospital of State University of Maringá.

**Funding Information**

We also thanks State University of Maringa and Federal University of Parana, Campus Palotina in special to Animal Science Pós-graduate Program for providing financial aid to conduct this study. The researchers are grateful for the support by Fundação Araucária – PR –BR.

**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.

**Silvia 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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