Identification of multi-resistant strains in bacteria associated with canine external ocular diseases

Esmeralda Delgado and **Sandra Subtil**

CIISA, Faculty of Veterinary Medicine, Ulisboa, Alameda da Universidade Técnica, 1300-477 Lisboa, Portugal

The identification of multi-resistant strains against some of the antibiotics used in therapeutics emphasizes the need for a correct use of antibiotics and the importance of previous bacterial culture and susceptibility testing in order to choose the appropriate therapy. This study highlights the most prevalent microorganisms occurring in canine ocular external diseases of the studied population (staphylococci, streptococci, Pseudomonas sp. and Pasteurella sp.). When antimicrobial agents are used incorrectly, for example during a too short period of time, in an inadequate dosage or for the wrong reasons, there is opportunity for bacteria to acquire resistance mechanisms. Although the development of multidrug resistance is a natural phenomenon, the inappropriate use of antimicrobial drugs contributes to the emergence and encourage further spread of multidrug resistance bacteria. It is a worrying fact that bacterial resistance to antimicrobials in veterinary medicine is growing worldwide. The consequences are serious as infections caused by resistant microorganisms do not respond to treatment, resulting in increased morbidity and mortality in both animals and humans.

Keywords: ocular external diseases; antimicrobial resistances; companion animals

1. Introduction

The resistance among various microbial species to different antimicrobial drugs has emerged as a cause of public health threat all over the world. Due to the advent of new resistance mechanisms and decrease in efficiency of treating common infectious diseases, it results in failure of microbial response to standard treatments.

In external ocular diseases in both man and animals the first therapeutical option is seldom recommended by an ophthalmologist… In fact, the chemist or the patient/owner usually chooses to apply a topical ophthalmic ointment containing an antibiotic as the first approach to any case of ocular inflammation, seldom necessary.

The ocular surface has a normal microbiota which plays a role in maintaining normal ocular health and preventing the overgrowth of potential pathogenic agents. [1, 2] These pathogenic agents have their origin from the skin ant upper respiratory tract. The most common microorganisms are Gram-positive bacteria, although sometimes Gram-negative organisms are isolated, which are thought to be transient and related with fungus in the environment. A large amount of bacteria is indicative of infection, although some dogs with palpebral malformations can accumulate substantial quantities of commensal bacteria in the conjunctival sac. [3]

Yet, these microorganisms can become potential pathogens when the cornea has a lesion, or in immunocompromised individuals. [4] Due to the variety of microorganisms present, an empiric therapy could be ineffective. 5 So it becomes evident that a culture and an AST provide useful information for the diagnostic and for choosing the most adequate antibiotherapy on a diversity of ocular diseases. [5]

When dogs have a severe or chronic conjunctivitis that does not respond to the initial treatment, a bacteriological analysis should be performed. [6, 7] Bacterial culture should not be the initial procedure when determining the cause of conjunctivitis, as it will probably reveal a microorganism present in normal conjuntival microbiota or a common pathogen. Several causes of conjunctivitis result in the overgrowth of normal microbiota or common pathogens, so the cause of conjunctivitis should not be attributed solely to bacteria. [5, 6]

Unlike cats, where most conjunctivitis are primary, in dogs the majority of infectious conjunctivitis are secondary to an underlying cause. The primary bacterial conjunctivitis in dogs is rare and usually occurs secondary to decreased tear production. In about 70 to 90% of normal dogs bacteria can be cultured from the conjunctival sac, and Staphylococcus sp. and other Gram-positive organisms are the most common isolates. [5] With regard to secondary conjunctivitis in dogs, the most frequent are follicular conjunctivitis, tear deficiency and allergic conjunctivitis. [8, 9] Successful treatment therefore depends on the identification and removal of the underlying cause. [5, 6]

It is also important to determine the antimicrobial susceptibility of pathogenic microorganisms associated with external ocular diseases, as indiscriminate use of antimicrobials for prophylaxis and treatment of minor infections affect the availability of antimicrobial drugs for serious diseases. [10]

The goals of this study were to evaluate the ocular flora and antimicrobial susceptibility patterns of the isolated bacteria in 91 samples obtained from 76 dogs with external ocular diseases from the Lisbon region, highlighting the most prevalent microorganisms and identifying multiresistant strains against some of the antibiotics most commonly used in therapeutics.

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2. Material and methods

2.1 Case selection

Case records of 76 dogs with external ocular diseases were reviewed. The population on study was composed of female and male dogs from the Lisbon region, Portugal, with signals of external ocular disease, of diverse breeds and ages. Samples were collected between 2006 and 2012 and they were sent for bacteriological analysis to the Bacteriology Laboratory from the Faculty of Veterinary Medicine - University of Lisbon.

2.2 Medical records review

Incomplete medical records or negative bacterial cultures were considered exclusion criteria from our study population. In total, 91 samples obtained from these 76 canine patients were considered in the study.

2.3 Sample collection and Processing

Prior to sample collection, all the animals underwent a complete ophthalmic examination. Samples were collected separately for each eye in non-responsive, chronic or exuberant clinical cases of canine external ocular infections (Fig. 1). Sampling for bacteriological analysis was done using sterile swabs rubbed gently on the lower conjunctival fornix sac or on the cornea prior to application of topical anesthetic or midriatic agents (Fig. 2). Given the existence of normal microbiota on the ocular surface, touching inadvertently tissues or regions with no obvious pathology was avoided.

The collected samples were smeared on Agar Mac Conckey plates (Oxoid, BioMérieux, France), Columbia Agar with 5% sheep blood a and inoculated in Brain Heart Infusion Broth (Liofilchem, Italy), and incubated in aerobic atmosphere at 37°C for 24 to 48 h. After observation and characterization of bacterial growth, suspect colonies were transferred to Columbia Agar with 5% sheep blood (Oxoid, BioMérieux, France) (Fig. 3) and the plates incubated in aerobic atmosphere at 37°C for 24 to 48 h. If no suspected colonies were observed, plates were kept in incubation for another 48 h before being considered negative.

2.4 Identification of isolates

The isolates were observed for morphological characteristics, submitted to rapid tests such as catalase test and/or oxidase test, and later identified by miniaturized biochemical tests, in particular the API system (Oxoid, BioMérieux, France).

2.5 Susceptibility Tests

The Susceptibility to Antimicrobials Tests were performed by the disk diffusion method, according to the standards established by the NCCLS (NCCLS, 2002) and by the CLSI (CLSI, 2008). 11

The drugs tested were FD 10 µg, NA 30 µg, AK 30 µg, AML 30 µg, AMC 30 µg, AMP 10 µg, CAR 100 µg, CL 30 µg, KF 30 µg, KZ 30 µg, CFP 75 µg, CTX 30 µg, CAZ 30 µg, CIP 30 µg, DA 2 µg, C 30 µg, DO 30 µg, ENR 5 µg, S 10 µg, CN 10 µg, IPM 10 µg, N 5 µg, OFX 5 µg, P 10 U, PRL 100 µg, SXT 25 µg, TE 30 µg and TOB 10 µg (Oxoid, Hampshire, United Kingdom).

Results described as "intermediate" susceptibility were considered resistant, both for statistical purposes and selection of antibiotherapy (Fig. 3).

Statistical analysis was done using descriptive analysis (Microsoft Office Excell, Microsoft Corporation, EUA).

Fig. 1 Infectious conjunctivitis with a large amount of purulent discharge in a female Bull Terrier aged 5 years, with a Schirmer Tear Test of 0 and a diagnosis of keratoconjunctivitis sicca. The patient was bilaterally affected.

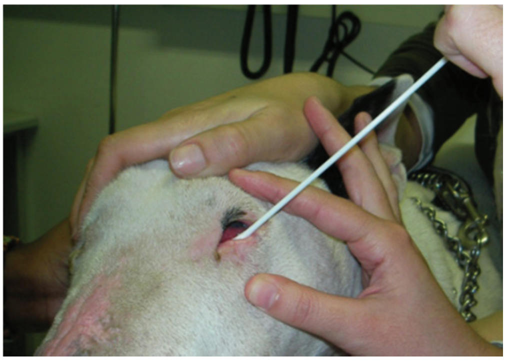

Fig. 2 Sampling for bacteriological analysis using a sterile swab rubbed gently on the conjunctival fornix, avoiding the contact with periocular skin. Same patient from Figure 1.

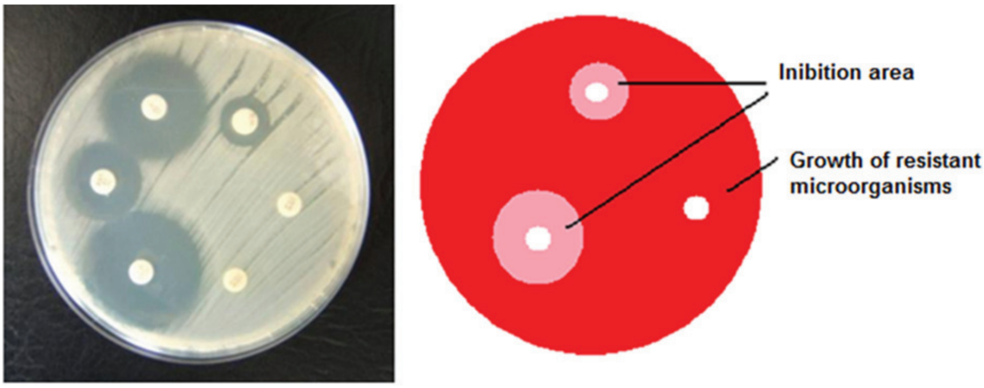

Fig. 3 On the right we can see a photo of a Susceptibility to Antimicrobials Test performed by the disk diffusion method, and on the left a scheme explanaining how to interpret the results.

3. Results

The isolated bacteria were mainly Gram-positive (76,9%) and the most common genus was Staphylococcus sp. (45%), followed by Streptococcus sp. (23%). The more frequent species were Staphylococcus aureus, Staphylococcus pseudintermedius and Streptococcus canis, in an equivalent percentage of 13,2%. From the Gram-negative isolated bacteria, the most frequent genus was Pseudomonas sp. (99%) and, within this, the specie Pseudomonas aeruginosa (6,6%). Another genus that appeared frequently was Corynebacterium sp. (6,6%), which are Gram-positive bacilli (Table 1).

Resistance to at least one drug was observed in 93,5% of the antibiograms. All staphylococci coagulase positive tested revealed resistance to amicacin, nalidixic acid, ciprofloxacin and ofloxacin (Table 2). All staphylococci coagulase negative demonstrated resistance to the association amoxicillin/clavulanic acid (Table 2).

All streptococci tested were resistant to fusidic acid, nalidixic acid, streptomicin, neomycin and tetraciclin (Table 3). Regarding antimicrobial resistance evidenced by the most frequent Gram-negative bacteria (Pseudomonas and Pasteurella genus), Pasteurella sp. (4,4%) showed resistance to ciprofloxacin, piperacilin, gentamicin and tobramicina (Table 4). Eight drugs (32%) were ineffective toward Pseudomonas sp., having this genus revealed total susceptibility to ciprofloxin and piperacilin (Table 4).

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Table 1 Bacterial Isolates found on the study (Nº - Number of samples with that isolate and % - from the total of 91 samples).

Isolate	**Nº**	**%**
Veilonella sp.	1	1,1
Arcanobacterium haemolyticum	1	1,1
C. ulcerans	1	1,1
Corynebacterium sp.	6	6,6
Acinetobacter lwoffi	1	1,1
E. coli	1	1,1
Enterobacter sp.	1	1,1
Klebsiella pneumoniae	2	2,2
Burkholderia cepacia	2	2,2
Pseudomonas aeruginosa	6	6,6
Pseudomonas fluorescens	2	2,2
Pseudomonas stutzeri	1	1,1
Pasteurella pneumotropica	1	1,1
Pasteurella sp.	2	2,2
Pasteurella multocida	1	1,1
Staphylococcus pseudintermedius	12	13,2
Staphylococcus aureus	12	13,2
Staphylococcus β-hemolítico	1	1,1
Staphylococcus auricularis	1	1,1
Staphylococcus sp.	8	8,8
Staphylococcus chromogenes	4	4,4
Staphylococcus epidermidis,	1	1,1
Staphylococcus haemolyticus	1	1,1
Staphylococcus simulans	1	1,1
Streptococcus canis	12	13,2
Streptococcus sp.	6	6,6
Streptococcus mitis	1	1,1
Enterococcus faecalis	1	1,1
Aerococcus viridans	1	1,1
TOTAL	91	100

Table 2 Antimicrobial Resistance evidenced by the staphylococci coagulase positive and staphylococci coagulase negative bacteria.

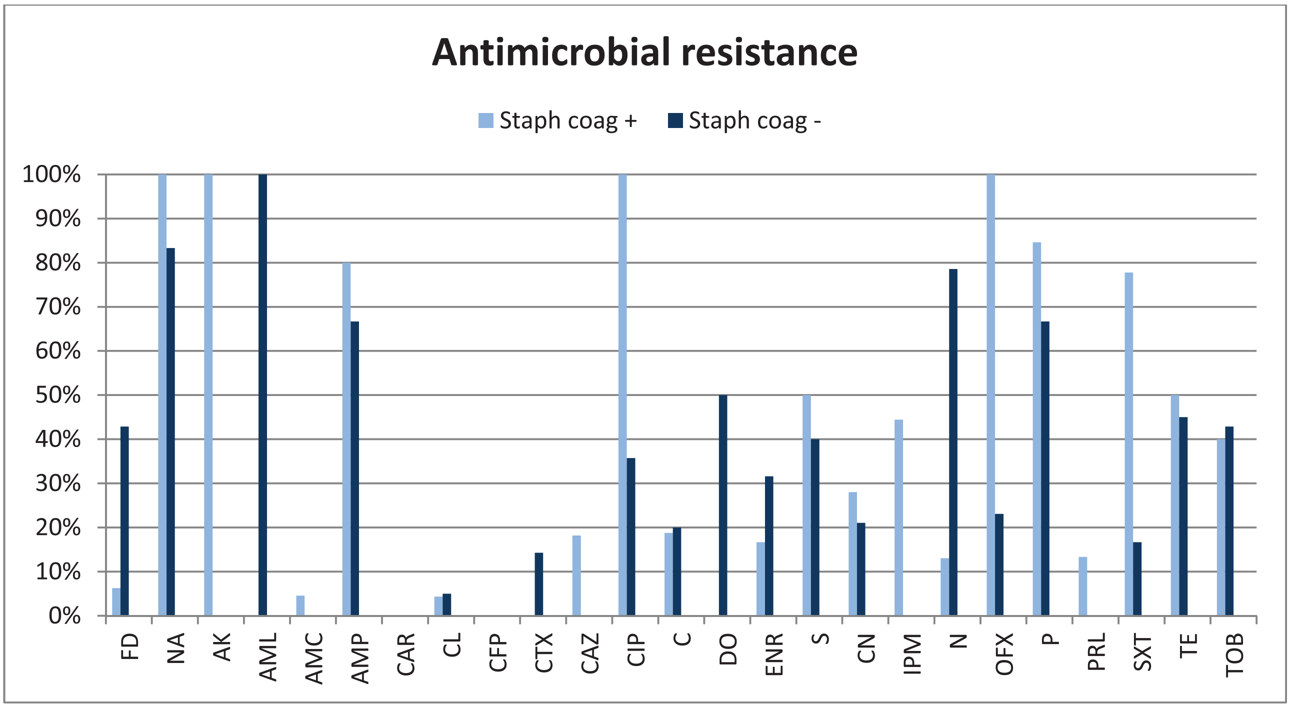

Table 3 Antimicrobial Resistance evidenced by bacteria of streptococcus genus.

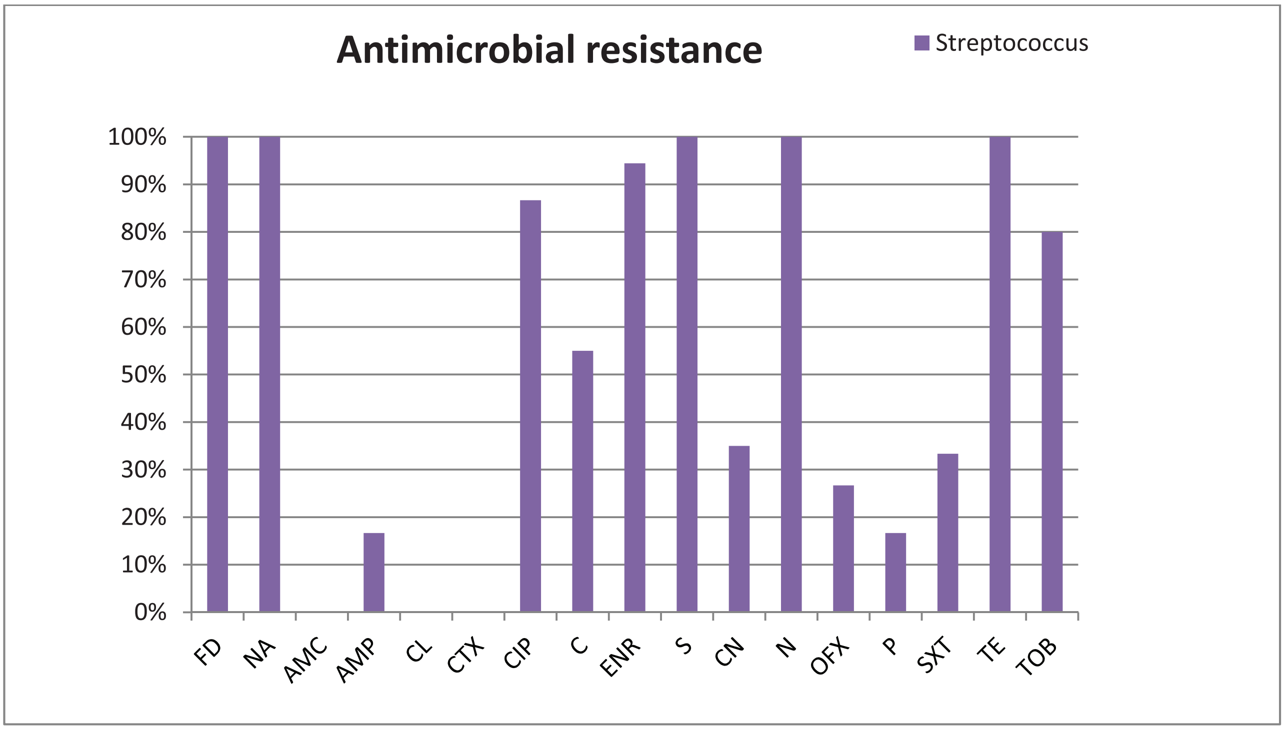

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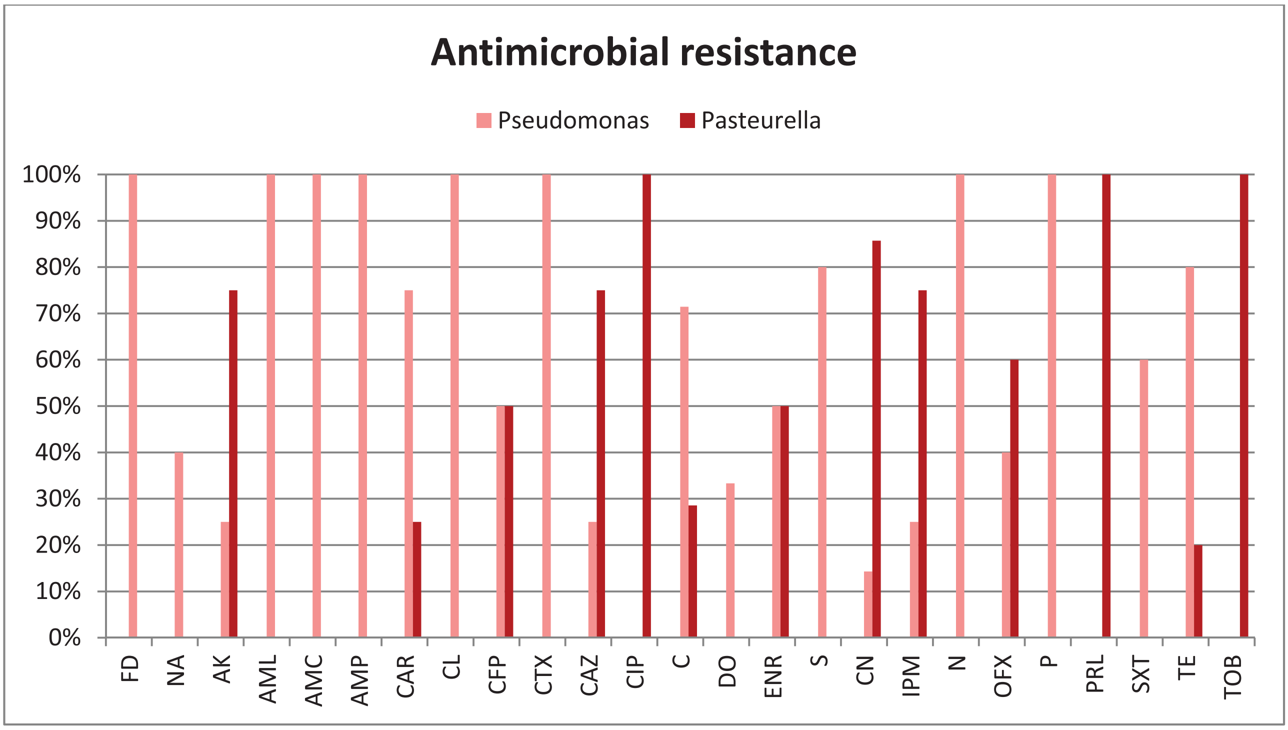
Table 4 Antimicrobial Resistance evidenced by most frequent Gram-negative bacteria (Pseudomonas and Pasteurella genus).



4. Discussion

The predominant genus found in this study was Staphylococcus sp. (45%), followed by Streptococcus sp. (23%). The more frequently isolated species were Staphylococcus aureus, Staphylococcus pseudintermedius and Streptococcus canis, (13,2%). From the Gram-negative isolated bacteria, the most frequent genus was Pseudomonas sp. (9,9%) and, within this, the specie Pseudomonas aeruginosa (6,6%). The results found in this study are in agreement with the available literature. [1, 2, 5, 12, 13]

The variation on the frequency of isolation of some bacterial genera and/or species is related to several factors affecting the individual prevalence of certain microorganisms. These include climate and location, season, the environment in which the animal lives and sampling and culture techniques. [14, 15, 16] However, all studies agree on the predominance of Gram-positive bacteria, 4,13 and within our results we found them on a percentage of 76.9%. As observed by Gerding (1988) and Murphy (1978), the most common isolates in our study were Staphylococcus spp. (45%) and Streptococcus spp. (23%). [1, 18] Other studies also report the genus Staphylococcus sp. as the most frequent, with percentages varying from 33% to 58%. [12, 13, 17] Staphylococci coagulase positive (25%) were the predominant group of microorganisms, which was also verified by the studies of Murphy et al. (1978); [18] Gerding et al. (1988); [1] Wang et al. (2008); [13] and Morales et al. (2009). [19]

Our results also reveal that in the studied population, the most frequently isolated species were Staphylococcus aureus, Staphylococcus pseudintermedius and Streptococcus canis. In this parameter studies are not unanimous, which is due to the factors outlined above, not only the external environment of the animal, geographical location and climate, but also characteristics inherent to the animal, particularly race, age and sex, and harvesting and processing of samples techniques. [2, 15]

From the Gram-negative isolated bacteria, the most frequent genus was Pseudomonas sp. (9,9%), which is in accordance with other authors. [1] This genus is known for its resistance to several drugs and for being a particularly dangerous pathogenic agent, for both animals and humans. It can colonize ocular epithelium and, as soon as local defenses are compromised in some way, the bacteria can proliferate rapidly through the production of extracellular enzymes such as elastase, alkaline protease and exotoxin A, and cause a rapidly destructive lesion (keratomalacia) which can lead to perforation and loss of the eyeball in 24 hours. It is considered an ophthalmic emergency and treatment should be started immediately, using effective antimicrobials against this bacterial genus, including gentamicin, ciprofloxacin or extended-spectrum penicillins, and other therapeutic agents. [7, 20, 21, 22]

The AST is indicated for any bacterial pathogen that contributes to an infectious process and for which is required the use of an antimicrobial drug. This is especially important when the etiologic agent is suspected to have resistance mechanisms to most commonly used antimicrobials (e.g. Pseudomonas aeruginosa), and also in studies concerning the epidemiology of resistance to antibacterial drugs and studies on new antibacterial drugs. [10, 11] Prolonged treatment with an inappropriate antimicrobial may result in overgrowth of pathogenic bacteria, yeasts and fungi. [10] Culture and performance of an AST is therefore important for successful treatment.

The antibiotics more frequently used in the treatment of external ocular infections are chloramphenicol, gentamicin, tobramycin and tetracycline as well, although this is mainly used in cats. [23] The present study demonstrates high rates of resistance against some of the antibiotics used in therapeutics, in particular tobramycin (80% of Streptococcus and 100% of Pasteurella are resistant to tobramycin), and tetracycline (100% of Streptococcus and 80% of Pseudomonas are resistant to tetracycline). Both chloramphenicol and gentamicin showed good efficacy in vitro against the bacterial isolates, which is consistent with the literature. [8, 20, 23, 24]

This surprising resistance pattern of Pasteurella spp. to tobramycin might be due to the inappropriate use of this drug as a first line antibiotic in ocular external infections.

The antimicrobial of first choice indicated in literature is chloramphenicol, which may be used before obtaining the culture and susceptibility results. [23, 24] After these results it is considered to maintain or change the treatment if there is evidence of antimicrobial resistance or intermediate susceptibility on AST. The antimicrobials of second choice are tobramycin and gentamicin, usually effective against Pseudomonas genus, so feared due to the high rate of resistance to antimicrobials. [23, 24]

In fact, in our study all isolates of Pseudomonas genus showed resistance to eight of the antimicrobials tested: fusidic acid, amoxicillin, amoxicillin / clavulanic acid, ampicillin, cephalexin, cefotaxime, penicillin and neomycin. Papich (2001) states that cephalosporins of second and third generation are not active against Pseudomonas aeruginosa, but there may be susceptibility to fluoroquinolones, aminoglycosides, or extended-spectrum penicillins such as ticarcillin or piperacillin. [20] In our results we observed that ciprofloxacin and piperacillin were 100% effective against Pseudomonas genus.

Resistant strains are selected after extensive use of a drug, mainly due to treatments implemented without prior bacterial culture and AST. The identification of multidrug resistance reinforces the importance of conducting these tests when prescribing medication for dog ocular external diseases, especially if they are chronic or exuberant. We therefore recommend treating each individual case according to the results of culture and susceptibility in order to choose the most appropriate therapy and avoid selection of resistant strains.

However, we may argue the use of topical antibiotics may have little selective pressure on the microbiome of dogs, particularly intestinal bacteria.

Nevertheless it is a worrying fact that bacterial resistance to antimicrobials in veterinary medicine is growing worldwide. The consequences are serious as infections caused by resistant microorganisms do not respond to treatment, resulting in increased morbidity and mortality in both animals and humans. [25] When antimicrobial agents are used incorrectly, for example during a too short period of time, in an inadequate dosage or for the wrong reasons, there is opportunity for bacteria to acquire resistance mechanisms. [25, 26, 27, 28] Several scientific publications describe a relation between antimicrobial used in animals and the emergence of resistance in bacterial strains of importance in human and animal pathology. [25, 26, 27, 28]

Antimicrobial resistance is a public health issue because we cannot forget that bacteria present in animals play a role as reservoirs of resistance genes that can cause disease in humans. [25, 26, 27, 28] Thus, the rational and safe use of antimicrobial agents in ocular diseases is essential to protect human and animal health against infectious diseases.

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