Conjunctival Microbiota Susceptibility to Antibacterials in Dogs

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Keywords: dog, conjuntivitis, susceptibility, antibacterials.

Abstract. Bacterial conjunctivitis is the most common ocular pathology in veterinary medicine. The aim of this study was to identify the conjunctival microbiota in dogs with conjunctivitis and to evaluate the antimicrobial susceptibility of the isolated strains. The research was performed at Dr. L. Kriauče-liūnas Small Animal Clinic of Lithuanian University of Health Sciences Veterinary Academy (LUHS VA) in 2018. Samples were tested at the microbiology laboratory of Microbiology and Virology Institute, LUHS VA. Dogs (n = 31) with symptoms of conjunctivitis were selected for primary ophthalmological assessment and research. Samples for bacterial testing were collected from the conjunctival sac. Susceptibility to antibiotics was tested using the Kirby-Bauer disc diffusion method. The investigation revealed that the most frequently isolated bacteria from the conjunctival sac were Gram+ bacteria (89.5%). The identifed bacteria belonged to the families Staphylococcaceae, Corynebacteriaceae, Enterobacteriaceae and Bacillaceae. The most efficient antibacterials against Staphylococcaceae family bacteria were represented by amoxicillin, cephalexin, Corynebacteriaceae by amoxicillin, amoxicillin/clavulanate, enrofloxacin, gentamicin, tetracycline, trimethoprim/sulfamethoxazole compound, and Bacillaceae by amoxicillin, amoxicillin/clavulanate, cephalexin, cefoxitin, enrofloxacin, erythromycin and gentamicin.

Introduction

Vision is the most important sense that provides survival superiority for many animals. Eye diseases are common in humans and animals. Therefore, the supply of medications is vast, but only in human medicine. In veterinary medicine, due to the lack of other options, medications designed for human use are the treatment of choice. However, not all human medications are suitable for animals: some of them are really effective, but irritate the eye. In such cases, a human patient simply waits until the irritation fades, whereas an animal immediately starts scratching and rubbing the eye, thus aggravating the inflammation and sometimes spreading it to surrounding tissues.

In a healthy animal, the epithelial barrier, mucin and tears help to rinse the palpebral conjunctiva. Antibacterial components in tears (lysozyme, beta lysine, lactoferrin, blood cells, IgA) protect the eye and its surrounding tissues from bacteria (Nadas et al., 2019).

Bacteria can be cultured from the conjunctival sac of about 70%–90% of normal dogs. The microbiota from the conjunctival sac is represented by mainly Gram+ species, with Staphylococcus spp. (57–70%), Streptococcus spp. (6–43%), Bacillus spp. (6–18%) and Corynebacterium spp. (30–75%) predominating. Predominant Gram– isolates that were recovered from the conjunctival sac in 7%–8% of normal dogs were Pseudomonas spp., Moraxella spp., and small quantities of Klebsiella spp., Neisseria spp. and Fusobacterium spp., and anaerobes were extremely rare. It was found that conjunctival microflora is also related to age, breed, climate, geology and geography and sampling method (Ollivier, 2003, Williams, 2017, Nadas et al., 2019).

Microbiota in the conjunctival sac can be divided into resident and opportunistic pathogenic organisms. Resident bacterial populations are usually isolated from bacteriologic samples of the canine conjunctiva in large numbers. They consist of noninvasive organisms that play an important homeostatic role by competing with pathogenic species for space and nutrients, and also by secretion of active substances that limit their ability to colonize the ocular surface (Williams, 2017).

In animals, conjunctivitis is usually caused by various infectious agents. Sometimes, there are mixed infections caused by several bacterial species or strains. Because non-pathogen bacteria can also be found in the conjunctival sac, the causative agent of inflammation is sometimes difficult to identify. Conjunctivitis is often accompanied by swelling and chemosis, resulting in blinking, sensitivity to light and pain. Animals start rubbing their eyes against surrounding objects, which may lead to traumatic corneal ulcers, and tissues become prone to infection. In dogs with conjunctivitis, the conjunctiva is irritated leading to a decrease in antibacterial enzymes (lactoferrin, lysozyme, and peroxidase). Moreover, the accompanying loss of epithelial integrity, depending on the degree and intensity of the trauma and tear film modifications, favors

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the colonization of ocular structures by opportunistic bacteria (Pereira et al., 2019).

Often bacterial conjunctivitis for dogs is secondary. The primary cause is usually mechanical irritation from eyelid abnormalities, such as entropion and ectropion, skin wrinkle contact with the cornea of the eyes in dogs of brachycephalic breeds, lash or hair abnormalities, such as ectopic cilia. Distichiasis or trichiasis can also be a cause of the conditions mentioned above. Impaired protective properties of the conjunctiva can lead to infection, and microorganisms of the normal flora can be potential pathogens. Another very probable cause of conjunctivitis is indiscriminate use or long-term application of antimicrobials because it may disrupt the normal conjunctiva microbiota balance and predispose to over-growth of pathogens. Most often, the ongoing infectious process is difficult to control (Athanasiou et al., 2018).

The common type of bacteria cultured from damaged eyes included *Staphylococcus* spp. (40%), *Streptococcus* spp. (64% – β -hemolytic streptococci, and 36% – α -hemolytic streptococci), *P. aeruginosa* (9%), *E. coli* (5%), and *Corynebacterium* spp. (4%). There are *Klebsiella* spp., *Neisseria* spp. and *Moraxella* spp. often found in the samples of the eyes, which are the saprophytic nasopharyngeal microbiota (Haghkhah et al., 2005, Marina et al., 2018, Pereira et al., 2019).

Medications containing gentamicin, chlortetracycline and cloxacillin have been already included in Lithuanian Veterinary Drug Register. Frequently, dog owners practice self-treatment and use eye medications without a veterinarian's prescription. Hence, bacterial strains resistant to antibacterials can develop. In the course of the last 10 years, this has been not only a Lithuanian, but a global problem as well. Although similar research has been performed in Lithuania (Kudirkienė et al., 2006), the number of conjunctivitis cases among dogs is increasing, including the difficult to treat ones. Due to the development of resistant bacteria strains, it is necessary to check the susceptibility of conjunctival isolates to drugs that are used in small animal veterinary medicine in Lithuania.

Many strains of bacteria can be isolated from the conjunctiva. Therefore, a question arises: does the activity spectrum of 3 antibiotic groups ensure the effective treatment of infections caused by Gram+ and Gram- bacteria?

Considering the importance of bacterial conjunctivitis in dogs, the aim of the study was to identify the microorganisms present in dogs with conjunctivitis and evaluate the antimicrobial susceptibility to antibacterials of the identified bacterial species.

Materials and Methods

The research was performed at Dr. L. Kriaučeliūnas Small Animal Clinic of Lithuanian University of Health Sciences Veterinary Academy in 2018. For the study purposes, 31 dogs diagnosed with conjunctivitis were evaluated. Samples for bacteriological testing were collected from the conjunctival sac. Before sampling, a full ophthalmic examination was carried out. The research was performed following the legislation acts of the European Union and the Republic of Lithuania.

Commercial bacteriological swabs were used to sample from the affected eyes into disposable tubes with a transport medium (Transwab, United Kingdom). Samples were tested at a microbiology laboratory of Microbiology and Virology Institute.

The clinically obtained material was disseminated on blood agar (CBA, EO Labs, United Kingdom). Plates with cultures were incubated at 35 ± 2 °C temperature for 24 hours in an aerobic and anaerobic environment. If there was no growth, plates were incubated for 48 hours more.

In case it was suspected that the number of bacteria in the sample was too small, the tested material was disseminated into a semi-liquid growth medium (Thioglycollate medium, Oxoid, United Kingdom), kept at +35°C temperature for 24 hours and then reintroduced into Colombia blood agar (EO LAB).

In order to identify the genus of staphylococci, the grown cultures were stained by Gram; their growth on Blood and Mannitol salt agar (Mannitol Salt Agar, Liofilchem, Italy) was evaluated. A catalase test was performed. In order to identify Staphylococcus species, identifying systems STAPH ID (Microgen, United Kingdom) were used. The results were read using computer program MID Ver1.2. Enterobacterales were identified by their colonial morphology, Gram's staining technique, typical growing characteristics on Mac-Conkey agar (Liofilchem, Italy), and SIM medium, where bacteria mobility, hydrogen sulfide, and indole production were evaluated. Preliminary identification of Corynebacteriaceae and Bacillaceae was performed based on rapid growth on blood agar and morphological properties stained by Gram. Corynebacteriaceae -Gram+ rods are seen in pairs in a smear. Bacillaceae - Gram+ spore rods.

Susceptibility to antibiotics was determined using the Kirby-Bauer disc diffusion method (EUCAST, 2020). Antibacterial discs were used: amoxicillin (AML; 10 µg), amoxicillin/clavulanate (AMC; 30 µg), cephalexin (CL; 30 µg), cefoxitin (FOX; 30 µg), clindamycin (CD; 2 µg), enrofloxacin (ENR; 5 µg), erythromycin (E; 15 µg), gentamicin (CN; 10 µg), tetracycline (TE; 30 µg), and sulfametoxazole/trimethoprim compound (SXT; 30 µg).

Susceptibility of *Staphylococcus* to amoxicillin and amoxicillin/clavulanic acid compound was evaluated according to susceptibility to cefoxitin and penicillin. The results were evaluated based on EUCAST (European Committee on Antimicrobial Susceptibility Testing) 2020 guidelines for *Enterobacterales, Staphylococcus* spp.

Enterobacterale susceptibility to tetracycline was evaluated based on CLSI M100 standard (CLSI

M100-ED29:2019). Susceptibility to enrofloxacin was evaluated according to CLSI VET08 standard (CLSI VET08 ED4:2018). *Corynebacteriaceae* susceptibility to tetracycline and clindamycin was evaluated according to EUCAST standard (EUCAST, 2020). Susceptibility to other antibiotics was evaluated by marginal values for *Staphylococcus* spp. *Bacillaceae* susceptibility was determined based on susceptibility/resistance criteria for *Staphylococcus* because there are no established clinical breaking points using the disc method.

Results

Gram+ organisms (89.5%) were more common than Gram– organisms in dogs with conjunctivitis. In a total of 31 samples, there were 57 isolates; the most commonly isolated organisms were *Staphylococcaceae* (64.9%), followed by *Corynebacteriaceae* (15.8%), *Enterobacterales* (10.5%), and *Bacillaceae* (8.8%).

In the samples where bacteria of the family *Staphylococcaceae* were isolated coagulase-negative species were predominant (*Staphylococcus* spp. 73.0%, *Staphylococcus chromogenes* 2.7%), followed by coagulase-positive species (*Staphylococcus aureus* 18.9%, *Staphylococcus intermedius* 5.4%). *Escherichia coli* (33.3%), *Proteus mirabilis* (33.3%), and *Enterobacter* spp. (33.4%) were identified in the samples where bacteria of the family *Enterobacterales* were isolated.

Bacterial associations due to a chronic infectious process were identified in 61.2% of the samples; the most common polymicrobial infection was represented by the association between two different pathogens (41.9%), followed by three pathogens in 16.1%, and four pathogens in 3.2% samples (Table 1). Infections caused by only one pathogen were found in 38.8% of cases.

Amoxicillin and cefalexin showed the greatest efficacy against the *Staphylococcaceae* family bacteria (91.2%), and the least susceptibility was established to amoxicillin/clavulanate (67.6%) (Figure 1). The susceptibility of the identified species of staphylococci to antibacterials differed from the average susceptibility of microorganisms of the *Staphylococcaceae* family (Figure 2). The most efficient antibacterials against *Staphylococcus aureus* were represented by gentamicin, enrofloxacin, clindamycin, and tetracycline (100%), followed by cephalexin, cefoxitin, erythromycin, amoxicillin, and amoxicillin/clavulanate (85.7%). The most efficient antibacterials against *Staphylococcus intermedius* were represented by amoxicillin, amoxicillin/clavulanate, cephalexin, tetracycline, clindamycin, and sulfamethoxazole/trimethoprim (100%), followed by cefoxitin, enrofloxacin, erythromycin, gentamicin, and clindamycin (50%). The most efficient antibacterials against *Staphylococcus chromogenes* were represented by cephalexin, cefoxitin, clindamycin, enrofloxacin, erythromycin, gentamicin, tetracycline and amoxicillin, and sulfamethoxazole/trimethoprim (100%), while and *Staphylococcus chromogenes* was resistant to amoxicillin/clavulanate.

Amoxicillin and amoxicillin/clavulanic acid compound showed the greatest efficacy against the *Corynebacteriaceae* family isolates (100%), and the least susceptibility was established to clindamycin (33.3%).

Amoxicillin, tetracycline, amoxicillin/clavulanate, enrofloxacin, gentamicin, and sulfamethoxazole/trimethoprim showed the greatest efficacy against the *Enterobacterales* isolates (100%), and the least susceptibility was established to cephalexin and cefoxitin (83.3%) (Figure 2). *Proteus mirabilis* isolates were susceptible to all tested antibacterials (100%). Amoxicillin, amoxicillin/clavulanate, tetracycline, gentamicin, and sulfamethoxazole/trimethoprim showed the greatest efficacy against the *E. coli* isolates (100%), followed by cephalexin (50%).

Amoxicillin, amoxicillin/clavulanate, cephalexin, enrofloxacin, gentamicin, and erythromycin showed the greatest efficacy against the *Bacillaceae* family bacteria (100%), and the least susceptibility was established to tetracycline (40%) (Figure 3).

Discussion

The diagnosis and treatment of ocular diseases in dogs are influenced by clinical examination of the eye and the identification of the ocular microbiota. In some cases, empirical treatment can be applied: antibacterial medications are then prescribed suspecting what the causative agent may be and anticipating its susceptibility. Therefore, it is important to know the susceptibility of bacteria that prevail in the region/ country, the probability of developing resistance, and its consequences.

The results of our study showed that of all tested samples from the conjunctival sac Gram+ bacteria were

Bacteria	Cases	Bacteria	Cases
Staphylococcus spp., Proteus mirabilis, Corynebacterium spp.	1	Staphylococcus spp., S. intermedius, Corynebacterium spp.	1
Staphylococcus spp., S. aureus	4	Staphylococcus spp., Proteus mirabilis, Corynebacterium spp, S. intermedius	1
Staphylococcus spp., S. aureus, S. chromogenes	1	Enterobacter spp., Bacillus spp., Staphylococcus spp.	1
Staphylococcus spp., Corynebacterium spp.	4	E.coli, Staphylococcus spp.	1
Staphylococcus spp., Bacillus spp.	3		

Table 1. Isolates from mixed bacterial infections



Fig. 1. Susceptibility of *Staphylococcacea* ir *Corynebacteriaceae* family bacterial isolates to antibacterials AML – amoxicillin, AMC – amoxicillin/clavulanate, CL – cephalexin, FOX – cefoxitin, CD – clindamycin, ENR – enrofloxacin, E – erythromycin, CN – gentamicin, TE – tetracycline, SXT – sulfamethoxazole/trimethoprim.



Fig. 2. Susceptibility of the coagulase-positive (S. aureus and S. intermedius) and coagulase-negative
S. chromogenes and Staphylococcus spp.) staphylococcal isolates to antibacterials (AML – amoxicillin,
AMC – amoxicillin/clavulanate, CL – cephalexin, FOX – cefoxitin, CD – clindamycin, ENR – enrofloxacin,
E – erythromycin, CN – gentamicin, TE – tetracycline, SXT – sulfamethoxazole/trimethoprim.



Fig. 3. Susceptibility of *Enterobacterales* and *Bacillaceae* bacterial isolates to antibacterials AML – amoxicillin, AMC – amoxicillin/clavulanate, CL – cephalexin, FOX – cefoxitin, CD – clindamycin, ENR – enrofloxacin, E – erythromycin, CN – gentamicin, TE – tetracycline, SXT – sulfamethoxazole/trimethoprim.

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□ Enterobacterales ■ Bacillaceae

isolated in 89.5% of cases. Different study reports show a high prevalence of Gram+ bacteria in the conjunctival sac of dogs with conjunctivitis, and it ranges from 56.5% to 67% (Williams, 2017, Pereira et al., 2019). There are some data indicating that up to 44% of conjunctival microbiota consist of Gram- bacteria (Williams, 2017). These results suggest that both Gram+ and Gram- bacterial agents play an important role in the pathogenesis of dogs with conjunctivitis. Previous investigations of the bacterial types associated with conjunctivitis in dogs have shown that Gram+ isolates predominate: Staphylococcus spp. (64.8%), Streptococcus spp. (18.9%), Corynebacterium spp. (0.8-3.5%), and Bacillaceae (2.7-4.7%) (Nadas et al., 2019, Pereira et al., 2019). In our research, the most frequent isolates were Staphylococcus spp. (64.9%) and Corynebacterium spp. (15.8%). However, some authors (Williams, 2017, Das et al., 2019) say that staphylococci are isolated much less frequently, in only about 35.3% of cases. In our research, enterobacteria were isolated in 10.5% of cases, much more often than other authors have reported. The most frequently isolated Gram- bacteria are Enterobacterales (2.7-14.4%), as well as Pseudomonas spp. (2.7%) (Williams, 2017, Nadas et al., 2019). No Pseudomonas spp. were isolated in our research.

Since the eye surface contains not only one singlefamily of bacteria, infection is also caused by several families of isolates. Our research revealed mixed infections in 61.2% of cases. Research data worldwide show similar results ranging from 29.7% to 53.3%. *Staphylococcus* spp. is a predominant bacterium of this type of infections (Williams, 2017, Nadas et al., 2019). In our research, mixed infections were caused by staphylococci (29.4%), and staphylococci with corynebacterias (29.4%) were most frequently found. Less frequently mixed infections were caused by staphylococci together with *Bacillus* spp. (17.6%).

Amoxicillin/clavulanate totally inhibited the growth of *Corynebacteriaceae*, *Enterobacterales*, and *Bacillaceae* bacterial isolates, and showed a weaker growth-inhibiting effect on *Staphylococcaceae* family isolates (67.6%). Research data by Das et al. (2019) indicate that potentiated amoxicillin had a stronger inhibitory effect on the growth of *Staphylococcus* spp. by 24–26.4%. There are certain data that there is almost no resistance against amoxicillin/clavulanate and exceptions are relatively rare (Pereira et al., 2019).

Our research revealed that non-potentiated amoxicillin acted against conjunctival isolates in 91.2–100% cases. The lowest effect of amoxicillin on the growth of the *Staphylococcaceae* family isolates was revealed. This antibiotic is very popular in clinical practice and, unfortunately, is used not reasonably; hence, there is a possibility of developing bacterial resistance because staphylococci produce beta-lactamases that inhibit the action of antibiotics against beta-lactam bacteria.

Our research showed 83.3–100% of bacteria susceptibility to cephalexin. Cephalexin totally inhibited the growth of the *Bacillaceae* family bacteria, and was slightly weaker against the *Staphylococcaceae* family isolates (91.2%). Meanwhile, research data by Auten (2019) and Das et al. (2019) indicate that cephalexin showed the strongest inhibitory effect on the growth of *Staphylococcaceae* isolates from the dogs' eyes. Data showing lesser activity of cephalexin may possibly be due to frequent use of this antibiotic and developing resistance. The lowest inhibitory effect of cephalex-in on the growth of the *Enterobacterales* isolates was found.

Fluoroquinolones can penetrate the cornea and get into hyaloid and eye liquid (Chung et al., 2013). In our research, enrofloxacin inhibited the growth of staphylococci in 83.3% of cases. However, other authors report different data. Das et al. (2019) indicate that susceptibility of staphylococci to fluoroquinolones reaches 100%, while Pereira et al. (2019) report only a 62.5–69.3% effect. Kang et al. (2014) report relative resistance of *Staphylococcus* spp. to fluoroquinolones: only 38.8% of isolates were susceptible (Pereira et al., 2019).

Erythromycin showed a growth-inhibiting action against Gram+ isolates in 71.4–100% of cases. It is one of the safest antibiotics. Other authors report similar data: Awosile et al. (2018) report that susceptibility of staphylococci to erythromycin reaches 62.8– 90.7%. Most macrolides show a post-antibiotic effect because they penetrate into phagocytes (neutrophils, macrophages, fibroblasts): their antibacterial activity remains present even when therapeutic concentration decreases (Steel et al., 2012).

Gentamicin was active against isolated bacteria in 44.4–100% of cases. It showed the strongest growth-inhibiting action against *Enterobacterales* and *Bacillaceae* isolates. Its weakest growth-inhibiting action was against the *Corynebacteriaceae* family bacteria. Research of Pereira et al. (2019) reports that amino-glycosides can be used empirically or for preventive treatment of eye infections in dogs. The research revealed that staphylococci were susceptible in 78.4% of cases, slightly more than the research by Williams indicates, i.e., 63% (Williams, 2017). Similar results were obtained by Pereira et al. (2019); meanwhile, Das et al. (2019) indicate more marked susceptibility of staphylococci to gentamicin (85%).

Susceptibility of *Enterobacterales* isolates to gentamicin is not equal. Various studies showed that gentamicin inhibited *E. coli* growth in 100% of cases. Pereira et al. (2019) report it to be 62.5%, whereas *Proteus mirabilis* were susceptible only in 50% of cases (Williams, 2017). Other researchers' data indicate that *E. coli* was resistant in 80% of cases. However, the testing was performed in Taiwan, where gentamicin is the antibiotic of the first choice and is widely used. This might be the reason for such resistance (Williams, 2017), although similar results were obtained by Das et al. (2019). In this case, *E. coli* susceptibility to gentamicin was only 33%, while the growth of *Proteus mirabilis* was completely inhibited by gentamicin. Corynebacteria isolated from the conjunctiva of sick dogs demonstrated lesser susceptibility to gentamicin than the data by Pereira et al. (2019) and Williams (2017) indicate, where gentamicin demonstrated 100% growth-inhibiting activity. Our research showed that gentamicin totally inhibited the growth of *Bacillus* spp. and *Enterobacterales* isolates. This data correspond with the results reported by Williams (2017).

In our research, tetracycline showed a 40-100% inhibiting action against bacteria isolates. Tetracycline showed the most expressed growth-inhibiting effect against Enterobacterales isolates; the weakest effect was against Bacillaceae family isolates. It was determined that Staphylococcus intermedius, one of the most common bacteria found in dogs, is susceptible to tetracycline only in up to 38.6% of cases (Pereira et al., 2019). According to the data from Kang et al. (2014), 94% of Staphylococcus intermedius isolated from the conjunctiva of sick dogs were resistant to tetracycline. Our research revealed a comparatively good effect of tetracycline against S. intermedius and S. chromogenes because the growth of these bacteria were inhibited in 100% of cases. Meanwhile, Pereira et al.'s (2019) data show susceptibility of enterobacteria to tetracycline only in 45.8% cases. Data presented in the literature indicate that Corynebacterium spp. is very susceptible to tetracycline, i.e., 100% (Pereira et al., 2019), but our research showed only a 66.7% susceptibility rate.

Sulphametoxazole/trimethoprim compound affected conjunctival isolates in 60–100% of cases. The most expressed growth-inhibiting effect was noticed against *Enterobacterales* isolates; the weakest effect was against *Bacillaceae* family bacteria. Sulfonamides have a wide action spectrum: they act against most Gram+ bacteria. They perfectly penetrate into eye tissues, but

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many bacteria have become resistant to these synthetic substances; besides, sulfonamides have a very high pH and irritate the conjunctiva (Tačič et al., 2017).

The results of our research revealed that bacteria isolated from the conjunctival sac of dogs diagnosed with conjunctivitis are susceptible to most antibacterials. Based on these results, empirical treatment can be warranted, paying attention to the recommendations regarding choosing first choice and reserved medications. Only unsuccessful treatment would require bacteriological tests that would help to adjust treatment.

Conclusions

- 1. Gram+ bacteria were most frequently isolated from the conjunctiva of dogs diagnosed with conjunctivitis (89.5%).
- 2. The best growth-inhibiting effect was demonstrated as follows: against *Staphylococcoceae* family by amoxicillin and cephalexin (91.2%); against *Corynebacteriaceae* by amoxicillin and amoxicillin/clavulanate (100%); against *Enterobacterales* by amoxicillin, amoxicillin/clavulanate, enrofloxacin, gentamicin, tetracycline, and sulphamethoxazole/ trimethoprim compound (100%); and against *Bacillaceae* by amoxicillin, amoxicillin/clavulanate, cephalexin, enrofloxacin, erythromycin, and gentamicin (100%).
- The least susceptible *Staphylococcoceae* isolates were to amoxicillin/clavulanic acid compound (67.4%); *Corynebacteriaceae* to clindamycin (33.3%), *Enterobacterales* to cephalexin and cefoxitin (83.3%); and *Bacillaceae* to tetracycline (40%).
- Medications included in Lithuanian Veterinary Drugs Registry are sufficient to effectively treat eye diseases in dogs caused by Gram+ and Grambacteria.

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Received 9 March 2020 Accepted 25 May 2020

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