UNIVERSIDADE DO OESTE PAULISTA
UNOESTE
PRESIDENTE PRUDENTE - SP

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

PERFIL DA SENSIBILIDADE MICROBIANA DE BACTÉRIAS ISOLADAS NOS OLHOS DE CÃES COM CERATOCONJUNTIVITE SECA

CAROLINA SILVA GUIMARÃES PEREIRA

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Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal – Área de concentração: Fisiopatologia Animal

Orientador:
Profa. Dra. Sílvia M. C. Franco Andrade

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2016

636.089 77
P436p

Pereira, Carolina Silva Guimarães.
Perfil da sensibilidade microbiana de bactérias isoladas nos olhos de cães com ceratoconjuntivite seca / Carolina Silva Guimarães Pereira. – Presidente Prudente, 2016.
59 f.: il.

Dissertação (Mestrado em Ciência Animal) - Universidade do Oeste Paulista – Unoeste, Presidente Prudente, SP, 2016.
Bibliografia.
Orientador: Sílvia M. C. Franco Andrade.

1. Ceratoconjuntivite. 2. Cães. 3. Testes de Sensibilidade Microbiana. I. Título.

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Presidente Prudente, 06 de maio de 2016

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DEDICATÓRIA

Dedico este trabalho principalmente a minha mãe Claudia de Fátima Silva que sempre me apoiou e nunca negou ajuda nos momentos mais difíceis.
Em memória de meu pai Emanuel Guimarães Pereira, que sempre me amparou quando eu precisei e me apoiou nas minhas decisões.
Aos meus avós Wilson José da Silva e Fátima do Nascimento Silva que sempre me ampararam em todos os momentos.
Ao meu irmão Gabriel Silva Guimarães Pereira, que soube respeitar os meus momentos de estudo.
Ao meu namorado Gabriel Molinari de Mattos, que participou desse momento, por toda compreensão, amor e carinho.
Ao meu irmão de coração Luís Felipe da Costa Zulim, que participou desse projeto, pela amizade, pela compreensão e incentivos, fez esse aprendizado ser mais feliz.
A Ieda Maria Munhós Benedetti, uma segunda mãe para mim, exemplo de vida, de profissionalismo, sempre me incentivou a seguir em frente nos estudos.

AGRADECIMENTOS

A Deus, que sempre tive fé, em todos os momentos me deu força, coragem e perseverança.

A minha família, que entenderam as minhas ausências e sempre me apoiaram nessa caminhada.

A professora e orientadora, Drª. Sílvia Maria Caldeira Franco Andrade, pela oportunidade de aprendizado e crescimento, na sua forma simples e pura, pela seriedade de seus ensinamentos, pelo total apoio, compreensão, e, principalmente, por acreditar em mim.

Ao professor e co-orientador, Dr. Rogério Giuffrida, pela paciência e educação, me proporcionou ensinamentos, valores profissionais e pessoais.

Ao amigo-irmão, que durante esse trabalho formamos uma dupla, Luís Felipe da Costa Zulim, pela ajuda, companheirismo, paciência, incentivo para que seguíssemos em frente e os muitos momentos de alegria compartilhados.

Ao grupo de trabalho, Aline Gutierres Cruz, Aline da Silveira Batista, Bruna Toledo Duran Foglia, Hugo Benguella, Luis Felipe da Costa Zulim, Rogério Giuffrida, Sidenir Bráz e Sílvia Maria Caldeira Franco Andrade. Obrigada pela oportunidade da convivência, risadas e principalmente por ser fonte do meu crescimento profissional e pessoal durante o desenrolar da fase experimental. Espero que tenham aprendido tanto quanto eu com as dificuldades, divergências, risadas, e trabalho em conjunto.

[...] Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho [...]"

Dalai Lama

RESUMO

Perfil da Sensibilidade Microbiana de Bactérias Isoladas nos Olhos de Cães com Ceratoconjuntivite Seca

Objetivou-se avaliar o perfil de sensibilidade microbiana de bactérias isoladas dos olhos de cães com ceratoconjuntivite seca (CCS). Foram avaliados 65 cães (n=65) diagnosticados com CCS e 30 cães (c=30) hígidos para o grupo controle. Foram coletados *swabs* conjuntivais e realizados os exames microbiológicos cultura em aerobiose, antibiograma e concentração inibitória mínima (CIM) para os antibióticos cloranfenicol, tobramicina, ofloxacina e moxifloxacina. Com relação à sensibilidade dos antibióticos testados, a polimixina B, a tobramicina e o cloranfenicol obtiveram maior percentual, enquanto que a tetraciclina o menor percentual. Com relação aos resultados da CIM, foram selecionadas as quinze cepas mais resistentes dos *Staphylococcus pseudintermedius* e as quinze cepas mais resistentes dos Gram-negativos. Para *S. pseudintermedius* a tobramicina expressou maior percentual de sensibilidade e a ofloxacina e moxifloxacina menor. Para Gram-negativos, ofloxacina e moxifloxacina apresentaram (100%) de sensibilidade, tobramicina (93,3%) e cloranfenicol (80%). Foram detectadas 3 linhagens de *S. pseudintermedius* multirresistentes, sendo um isolado sensível à cefazolina, outro a vancomicina e outro a polimixina B e amicacina. Em 100% dos animais tratados topicamente com os antibióticos selecionados pela sensibilidade, obteve-se a remissão da infecção bacteriana após 15 dias. As bactérias isoladas de olhos de cães com CCS possuem sensibilidade variável frente aos antibióticos testados, de acordo com a espécie considerada. A emergência de linhagens quinolona-resistentes de *S. pseudintermedius*, agente de prevalência mais alta detectada nesses olhos, reforça a necessidade de identificação da bactéria envolvida e perfil de sensibilidade microbiana, visto que a infecção secundária, pode ser um fator agravante e perpetuante da CCS.

Palavras-chave: olho seco, cães, antibiograma, disco-difusão, concentração inibitória mínima

ABSTRACT

Profile of Bacteria Microbial Sensitivity Isolated in Dogs with Eyes Keratoconjunctivitis Sicca

This study aims to evaluate the microbial sensitivity profile of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca (KCS). We evaluated 65 dogs (n=65) diagnosed with KCS and 30 healthy dogs (c=30) (control group). After diagnosis of KCS, conjunctival *swabs* were collected and the microbiological examinations performed under aerobic culture, antibiogram and minimum inhibitory concentration (MIC) for the antibiotics chloramphenicol, tobramycin, ofloxacin and moxifloxacin. With respect to the sensitivity to the tested antibiotics, polymyxin B, tobramycin and chloramphenicol obtained that highest percentages and tetracycline the lowest percentage. Regarding the results of the MIC, the fifteen most resistant strains of *Staphylococcus pseudintermedius* and the fifteen most resistant strains of Gram-negative were selected. For *S. pseudintermedius,* tobramycin demonstrated the highest percentage of sensitivity and ofloxacin and moxifloxacin the lowest. For Gram-negative, ofloxacina and moxifloxacina demonstrated (100%) of sensitivity, tobramycin (93,3%) and chloramphenicol (80%). Three multi-resistant strains of *S. pseudintermedius* were detected, one with isolated sensitivity to cefazolin, another to vancomycin and another to polymyxin B and amikacin. Remission from bacterial infection was achieved after 15 days in 100% of the animals topically treated with antibiotics selected according to the sensitivity. The bacteria isolated from the eyes of dogs with KCS presented variable sensitivity to the tested antibiotics, according to the species considered. The emergence of quinolone-resistant strains of *S. pseudintermedius*, with higher prevalence detected in the eyes, reinforces the need to identify the bacteria involved and antimicrobial susceptibility profile, as secondary infections, can be an aggravating and perpetuating factor of KCS.

Key-words dry eye, dogs, antibiogram, disk-diffusion, minimum inhibitory concentration

LISTA DE SIGLAS

ARVO	– Associação de pesquisa em visão e oftalmologia - Declaração para a utilização de animais na investigação oftalmológica e visual
CEUA	– Comissão de uso de animais em experimentação
CCS	– Ceratoconjuntivite seca
CFZ	– Cefazolina
CIM	– Concentração inibitória mínima
CIP	– Ciprofloxacina
CLO	– Cloranfenicol
DD	– Disco-difusão
DNA	– Ácido desoxirribonucleico
ETEST	– Avaliação da metodologia CIM
GEN	– Gentamicina
IC	– Intervalo de confiança
mm/min	– Milímetros por minuto
MO	– Microrganismo
NEO	– Neomicina_
NOR	– Norfloxacina
NT	– Não testado
OD	– Olho direito
OE	– Olho esquerdo
OFX	– Ofloxacina
P	– Nível de significância
POL. B	– Polimixina B
S	– Sensível
SRD	– Sem raça definida
TCLE	– Termo de consentimento livre e esclarecido
TET	– Tetraciclina
TF	– Teste de fluoresceína
TLS	– Teste lacrimal de Schirmer
TOB	– Tobramicina
TRB	– Teste de rosa bengala
TRFL	– Tempo de ruptura do filme lacrimal
µg/mL	– Microgramas por mililitros
IV	– Intravenosa
x/dia	– Vezes ao dia

SUMÁRIO

1. ARTIGO CIENTÍFICO

Perfil da sensibilidade microbiana de bactérias isoladas nos olhos de cães com ceratoconjuntivite seca

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Título Abreviado: Perfil sensibilidade microbiana bactérias isoladas CCS cães

RESUMO

Objetivos Avaliar o perfil de sensibilidade microbiana de bactérias isoladas nos olhos de cães com ceratoconjuntivite seca (CCS).

Procedimento Foram avaliados 65 cães diagnosticados com CCS e 30 cães hígidos (grupo controle). Após o diagnóstico da CCS foram coletados *swabs* conjuntivais e realizados os exames microbiológicos cultura em aerobiose, antibiograma e concentração inibitória mínima (CIM) para os antibióticos cloranfenicol, tobramicina, ofloxacina e moxifloxacina.

Resultados Com relação à sensibilidade dos antibióticos testados, a polimixina B, a tobramicina e o cloranfenicol apresentaram percentual mais alto, enquanto que a tetraciclina o mais baixo percentual. Com relação aos resultados da CIM, foram selecionadas as quinze cepas mais resistentes dos *Staphylococcus pseudintermedius* e as quinze cepas mais resistentes dos Gram-negativos no teste de antibiograma. Para *S. pseudintermedius* a tobramicina expressou maior percentual de sensibilidade e a ofloxacina e moxifloxacina menor. Para Gram-negativos, ofloxacina, moxifloxacina, tobramicina e cloranfenicol apresentaram excelente sensibilidade. Foram detectadas 3 linhagens de *S. pseudintermedius* multirresistentes, sendo um isolado sensível à cefazolina, outro a vancomicina e outro a polimixina B e amicacina. Em 100% dos animais tratados topicamente com os antibióticos selecionados pela sensibilidade, obteve-se a remissão da infecção bacteriana após 15 dias.

Conclusão As bactérias isoladas de olhos de cães com CCS possuem sensibilidade variável frente aos antibióticos testados, de acordo com a espécie considerada. A emergência de linhagens quinolona-resistentes de *S. pseudintermedius*, agente de prevalência mais alta detectada nos olhos, reforça a necessidade de identificação da bactéria envolvida e perfil de sensibilidade microbiana, visto que a infecção secundária, pode ser um fator agravante e perpetuante da CCS.

Palavras-chave olho seco, cães, antibiograma, disco-difusão, concentração inibitória mínima**.**

INTRODUÇÃO

A ceratoconjuntivite seca (CCS), ou olho seco, é uma oftalmopatia comum em cães que ocorre geralmente pela deficiência do componente aquoso do filme lacrimal pré-corneano, ou devido à modificação da qualidade e/ou diminuição da estabilidade da lágrima. A CCS possui várias etiologias, sendo a imunomediada mais prevalente. [1-5]

A terapia tópica da CCS consiste principalmente no uso de imunossupressores como ciclosporina, tacrolimus ou pimecrolimus e substitutos lacrimais associados ao uso de antimicrobianos para tratamento de infecções secundárias, incluindo gentamicina, tobramicina, cloranfenicol, ofloxacina, moxifloxacina e gatifloxacina, e anti-inflamatórios tópicos, mucolíticos e estimulantes parassimpatomiméticos da secreção lacrimal. [2,5]

A superfície ocular é colonizada por bactérias saprofíticas que, conjuntamente, com fatores humorais, inibem o estabelecimento de microrganismos patogênicos. Em cães hígidos estão presentes na superfície ocular, principalmente, bactérias Gram positivas, com perfil de espécies que varia em razão da localização geográfica, técnica de cultivo da secreção lacrimal, contato entre animais, densidade populacional e estações do ano.[6-8]

Em olhos que sofrem uma injúria externa, dependendo do grau e intensidade do trauma, ou que apresentam modificações no filme lacrimal que leva à diminuição nas enzimas antibacterianas (lactoferrina, lisozima e peroxidase), desencadeiam-se processos inflamatórios superficiais na córnea e conjuntiva, com perda de integridade do epitélio, favorecendo o estabelecimento de bactérias e fungos oportunistas e colonização de estruturas oculares.[7]

Microrganismos com alta prevalência nas conjuntivites de cães incluem as espécies *Staphylococcus epidermidis, S. pseudintermedius, S. aureus, Streptococcus pneumoniae, E. coli, Streptococcus spp, Enterobacter* sp., e *Pseudomonas* sp.[8] Em cães com CCS, estes agentes frequentemente expressam resistência a vários princípios antimicrobianos utilizados na terapêutica. A identificação de multirresistências vem reforçar a importância do uso

racional dos antimicrobianos e da realização de culturas microbianas e testes de sensibilidade a antimicrobianos, para nortear a escolha da terapêutica apropriada.[8-10]

A terapia antimicrobiana para doenças oculares, principalmente em conjuntivites, ceratites e ulceras de córnea, preferencialmente é realizada por via tópica por meio de colírios antimicrobianos, sendo os mais utilizados em medicina veterinária, cloranfenicol, aminoglicosídeos (neomicina, gentamicina, tobramicina), tetraciclinas, quinolonas de segundo geração (ofloxacina, ciprofloxacina) e de quarta geração (gatifloxacina e moxifloxacina). [5,9]

A escolha destes antimicrobianos deve ser respaldada em testes de sensibilidade. O método de disco-difusão, de Kirby-Bauer descrito em 1966, é uma técnica de fácil execução e de baixo custo e está amplamente disponível em vários laboratórios de diagnóstico.[10] Alternativamente, a determinação da concentração inibitória mínima (CIM), menor diluição de um fármaco capaz de inibir o crescimento de microrganismos, vem ganhando notoriedade, devido à boa sensibilidade, facilidade de execução, alta reprodutibilidade e alta confiabilidade dos resultados. [10]

Existem poucos estudos sobre o perfil de sensibilidade de agentes bacterianos envolvidos em infecções secundárias oculares em cães com CCS. Dessa forma, o objetivo deste estudo foi avaliar a sensibilidade microbiana pelo método de disco-difusão e determinação da concentração inibitória mínima (CIM) para bactérias isoladas na secreção ocular de cães com CCS.

MATERIAIS E MÉTODOS

O estudo foi realizado no Hospital Veterinário da Universidade do Oeste Paulista – UNOESTE, Presidente Prudente, SP. Foram avaliados 65 cães, sem predileção de raça, faixa etária e sexo, diagnosticados com CCS, cadastrados sob termo de autorização (Termo de Consentimento Livre e Esclarecido – TCLE) assinado pelos respectivos proprietários e

responsáveis pelo projeto, e para o grupo controle foram selecionados aleatoriamente 30 cães do canil de instituição de ensino, hígidos (avaliados por exame clinico e laboratorial) e sem problemas oftálmicos (avaliados pelo exame ocular com lâmpada de fenda, Teste Lacrimal de Schirmer, Teste de Fluoresceína, e Tempo de Ruptura do Filme Lacrimal). O estudo foi conduzido e aprovado conforme as normas de experimentação animal da Comissão de Ética no Uso de Animais (CEUA) da UNOESTE (protocolo n° 1802 e 1803) e está de acordo com as normas da ARVO (Association for Research in Vision and Ophthalmology – Statement for the use of animals in ophthalmic and visual research).

O diagnostico de CCS foi baseado em sinais clínicos e testes oftálmicos específicos que incluíram o teste lacrimal de Schirmer (TLS) e tempo de rompimento do filme lacrimal (TRFL) ou *Break Up Time* (BUT). Para a realização do TLS, uma tira de papel filtro de 0,5 cm (Teste de Schirmer Ophthalmos®) foi introduzida no saco conjuntival por um período de 1 min. Foram considerados cães positivos para CCS, os que apresentaram valores de TLS ≤ 10 mm/min. Para o teste do TRFL uma tira estéril de fluoresceína a 1% (Laboratório Ophthalmos, São Paulo, Brasil) embebida em 1 gota de solução fisiológica foi colocada em contato com o saco conjuntival. Após o cão realizar duas piscadas, a pálpebra foi imobilizada e observou-se a córnea com um biomicroscópio (lâmpada de fenda portátil) para determinar o TRFL. Dois testes sucessivos foram realizados para calcular-se um valor médio de TRFL. Valores médios de TRFL ≤ 10 seg foram considerados positivos para CCS.[11]

Para determinar a existência de úlceras de córnea, utilizou-se o teste de fluoresceína (TF), que consistiu em empregar uma tira estéril de fluoresceína a 1% (Laboratório Ophthalmos, São Paulo, Brasil) embebida em uma gota de solução fisiológica e encostá-la no saco conjuntival. A presença de úlceras de córnea foi evidenciada após exposição a luz com áreas esverdeadas. Adicionalmente, empregou-se o Teste de Rosa Bengala (TRB) utilizado o colírio de Rosa Bengala a 1% (Laboratório Ophthalmos, São Paulo, Brasil), com instilação

prévia de colírio anestésico, para coloração de células da córnea e conjuntiva desvitalizadas pela CCS. Foi considerado positivo para presença de células desvitalizadas pela CCS quando áreas da córnea ou conjuntiva se coraram em rosa. [11]

Após o diagnóstico da CCS, foram coletadas amostras da secreção ocular da conjuntiva inferior de ambos os olhos com *swabs* estéreis de algodão. As amostras foram semeadas em placas de Petri contendo ágar sangue bovino desfibrinado a 5% e ágar MacConkey. As placas semeadas foram incubadas em aerobiose na temperatura de 37°C, durante 24-48 horas. Colônias bacterianas isoladas foram classificadas segundo características morfotintoriais e bioquímicas.[12] Para os testes de sensibilidade, após o isolamento e a identificação, os isolados foram semeados em caldo cérebro coração e incubados a 37° C por 24 horas em aerobiose. Após este período, os caldos foram diluídos em solução fisiológica até atingirem turvação correspondente à escala 0,5 de Mac Farlland. A seguir, com auxílio de um *swab* estéril, uma alíquota do caldo foi semeada superficialmente em placas de ágar Mueller-Hinton.

Após o período de 30 minutos, necessário para a absorção do inoculo, foram depositados uniformemente sobre a superfície do ágar, discos de papel-filtro (Cecon, São Paulo, Brasil) contendo os seguintes agente antibacterianos: Cefazolina (30mcg), Ciprofloxacina (5mcg), Cloranfenicol (30mcg), Gentamicina (10mcg), Neomicina (30mcg), Norfloxacina (10mcg), Ofloxacina (5mcg), Polimixina B (300mcg), Tetraciclina (30mcg), Tobramicina (10mcg) e Vancomicina (30mcg) (Fig. 1 A, B). Após incubação de 18 horas, o diâmetro das zonas de inibição em torno dos discos foi mensurado com paquímetro e os resultados expressos em milímetros foram comparados com padrões internacionais para a interpretação de resultados.[12]

A concentração inibitória mínima para os isolados de maior relevância e potencialmente patogênicos para cães foram avaliados quanto à sensibilidade frente ao cloranfenicol, tobramicina, ofloxacina e moxifloxacina pelo método de E-test (Probac, São Paulo, Brasil).

Uma alíquota do caldo foi semeada superficialmente em placas de ágar Mueller-Hinton com auxílio de swab estéril. Após 30 minutos, tiras plásticas comerciais impregnadas com cloranfenicol (0.016 – 256 µg/mL), tobramicina (0.064 – 1024 µg/mL), ofloxacina e moxifloxacina (0.002 – 32 µg/mL), foram depositadas na superfície do ágar. Após incubação a 37°C por 24 horas, os pontos de inibição de crescimentos observados em torno das tiras, correspondente a concentração inibitória mínima, foram computados para cada agente (Fig. 1 C, D). Os padrões observados foram comparados com os pontos de corte padronizados pelo Clinical and Laboratory Standards Institute (2012)[12] para classificar os isolados como sensíveis, parcialmente sensíveis e resistentes. Os pontos de corte considerados para *S. pseudintermedius* foram: Cloranfenicol S = ≤ 8 µg/mL; moxifloxacina S = ≤ 0,5 µg/mL; ofloxacina S = ≤ 1 µg/mL e tobramicina S= ≤ 8 µg/mL e para gram negativos de cloranfenicol S = ≤ 8 µg/mL; moxifloxacina S = ≤ 0,5 µg/mL; ofloxacina S = ≤ 2 µg/mL e tobramicina S= ≤ 4 µg/mL.

As prevalências de infecções bacterianas oculares foram determinadas para cães com CCS e cães do grupo controle, estimadas por ponto e por intervalo com 95% de confiança. Os percentuais de cães positivos no grupo com CCS e controle foram comparados pelo teste binomial. A concordância entre os resultados dos testes de sensibilidade aferidas pelo E-test e disco-difusão foi aferida por meio da análise do coeficiente Kappa de concordância. Foi adotado o nível de significância de 5%.

RESULTADOS

Dos 65 cães diagnosticados com CCS, 36/65 (55%) foram fêmeas e 29/65 (45%) machos. Dentre as raças dos animais selecionados, as mais frequentes foram Lhasa Apso e sem raça definida (SRD) com 17 (26,2%) animais cada, seguido de Poodle com 10 (15,4%), Pinscher com 5 (7,7%), Yorkshire Terier com 3 (4,6%), Pitbull com 2 (3,1%) e outras 11 raças com 1,5%.

No grupo de animais com CCS, 59 de 65 cães (90,8%, IC95 = 83,7%-97,8%) apresentaram cultura positiva para pelo menos um dos olhos. No grupo controle, 11 de 30 cães (36,7%, IC95% = 19,4%-53,9%) apresentaram cultura positiva para pelo menos um dos olhos. Os percentuais de positividade foram estatisticamente diferentes entre grupo controle e com CCS (p <0,0001).

Todos os isolados provenientes do grupo controle foram classificados com *S. pseudintermedius.* Para animais com CCS, o perfil microbiológico consistiu de espécies bacterianas Gram-positivas e Gram-negativas (Tabela 1). Verificou-se que 7,5% dos microrganismos foram isolados somente em um dos olhos, enquanto que 92,4% dos isolamentos foram concordantes em ambos os olhos.

A sensibilidade dos agentes isolados pelo método de disco-difusão está descrita na Tabela 2. Foram selecionados para determinar a CIM pelo método E-test, quinze cepas de *S. pseudintermedius* e quinze de gram-negativos (onze cepas de *E. coli*, três cepas de *Enterobacter aerogenes* e uma cepa de *Citrobacter freundii*) (Tabela 3). Verificou-se alta concordância entre os métodos de disco-difusão e E-test para cloranfenicol, boa para ofloxacina e fraca para tobramicina (Tabela 4).

Cães com CCS com isolamentos positivos, caracterizando infecção ocular, foram medicados com respaldo dos testes de sensibilidade com colírios antimicrobianos. Em três (4,6%) dos 65 cães foram detectadas linhagens de *S. pseudintermedius* resistentes a nove ou mais princípios antimicrobianos, incluindo a oxacilina, caracterizando múltipla-resistência. Em face destes resultados, procederam-se novos testes de sensibilidade, nos quais verificou-se que um dos isolados (N4) foi sensível a cefazolina (Fig. 2 A, B), um (N5) a vancomicina (Fig. 2 C, D) e um (N13) a polimixina B e amicacina (Fig. 2 E, F). Em razão da ausência de preparações comerciais adequadas no mercado brasileiro, optou-se pela prescrição de

soluções oftálmicas manipuladas contendo estes princípios (Laboratório Ophthalmos®, São Paulo, Brasil). Em 100% dos animais medicados, obteve-se a remissão da infecção bacteriana após 15 dias de medicação contínua (1 gota, em ambos os olhos, 4 vezes ao dia).

DISCUSSÃO

A alta porcentagem de cães com CCS com culturas positivas (90,8%), em relação ao grupo controle (36,7%), reforça a importância da identificação do perfil de agentes bacterianos e sensibilidade frente a antimicrobianos para nortear critérios no tratamento nesta patologia. [13,14] Cães com CCS apresentam deficiência de filme lacrimal, seja na produção ou na evaporação, consequentemente, há diminuição nas enzimas antibacterianas (lactoferrina, lisozima e peroxidase), situação que favorece o estabelecimento de bactérias oportunistas da microbiota superficial dos cães no saco conjuntival, contribuindo para maior frequência de culturas positivas em relação aos cães sadios, o que corrobora com resultados observados.[2-7]

O patógeno de maior prevalência foi *S. pseudintermedius*, isolado de aproximadamente 67% das amostras pesquisadas. *S. pseudintermedius* é uma espécie coagulase-postiva, saprofítica da pele e mucosas de cães e que comumente coloniza olhos de cães hígidos.[15-18]

Observou-se predominância de microrganismos Gram negativos entéricos em relação a outros Gram negativos, com destaque para *Escherichia coli* que representou aproximadamente 11% das amostras isoladas, seguido de *Enterobacter aerogenes* (3%). Esses resultados são condizentes com outros estudos, em que *E. coli* representou 10% das amostras isoladas [18-19] *P. aeuruginosa*, microrganismo capaz de secretar enzimas que resultam na liquefação do estroma corneal com rápida evolução para ulceração,[18] apresentou baixa frequência em relação aos demais agentes, com sensibilidade a todos os antimicrobianos testados.

Quando se avaliou a sensibilidade dos agentes isolados pelo método de disco-difusão, a tobramicina, apresentou melhor efetividade do que as quinolonas testadas, ofloxacina e moxifloxacina. Os aminoglicosídeos podem ser alternativas adequadas para o tratamento empírico ou profilático em oftalmologias de cães, em comparação às quinolonas como ciprofloxacina, moxifloxacina e gatifloxacina, antimicrobianos aos quais os microrganismos descritos tem apresentado nos últimos dez anos, notável resistência. [18, 20,21]

No presente estudo, observou-se boa eficiência da Polimixina B e cloranfenicol. Estes resultados estão de acordo com o relatado por outros autores. O cloranfenicol é um antibiótico de amplo espectro e tem sido mais utilizado desde que surgiram *Staphylococcus* resistentes à meticilina, porém, devido aos efeitos tóxicos em seres humanos, o uso da droga tem sido limitada. [22]

Três cães que apresentaram linhagens de *S. pseudintermedius* resistentes a oxacilina, antimicrobiano considerado como importante marcador de multirresistência em infecções oculares de humanos.[20] Outros autores relatam também um aumento da resistência de *S. pseudintermedius* em infecções oculares em cães.[23-25] Nestes três casos de resistência observada no presente estudo, testes de sensibilidade adicionais revelaram que estes isolados apresentaram sensibilidade a dois antibióticos de uso exclusivo injetável no Brasil, no caso, vancomicina, antibiótico glicopeptídeo utilizado em infecções hospitalares e a cefazolina antibiótico do grupo das cefalosporinas.

Verificou-se menor eficiência da tetraciclina frente aos isolados, visto que 40,2% foram sensíveis a este antibiótico. Este resultado é condizente com outros estudos, que relataram que mais de 50% de linhagens de *S. pseudintermedius* isolados de patologias oftálmicas foram resistentes à tetraciclina.[26]

Com relação a sensibilidade dos agentes isolados pelo método E-test, verificou-se que a tobramicina possui boa efetividade (93,3%) frente a microrganismos Gram-negativos e Gram-positivos como *S. pseudintermedius*, o que condiz com os resultados observados em outros estudos.[27,28] De acordo com o E-test, as quinolonas testadas apresentaram baixa efetividade frente a *S. pseudintermedius*, com percentuais de resistências superiores a 80%. Considerando que moxifloxacina e ofloxacina são quinolonas de uso mais recente, comumente empregadas para tratamento de oftalmopatias em cães, os resultados sugerem possível emergência de linhagens resistentes em animais da região.[26]

Em comparação, os Gram-negativos apresentaram-se sensíveis as quinolonas testadas, o que sugere que a pressão de seleção sobre estes microrganismos é inferior a observada para *S. pseudintermedius*, com exceção de linhagens de *E. coli* multirresistentes comumente observadas em infecções urinárias.[29,30] Avaliou-se que existe excelente concordância entre resultados do E-test e Disco-difusão, para cloranfenicol (0.8421) e ofloxacina (0.7368), porém limitada para tobramicina (0.3284).

Conclui-se que as bactérias isoladas de olhos de cães com CCS possuem sensibilidade variável frente aos antibióticos testados, de acordo com a espécie considerada. A emergência de linhagens quinolona-resistentes de *S. pseudintermedius*, agente de prevalência mais alta nos olhos destes animais, reforça a necessidade de identificação da bactéria envolvida e perfil de sensibilidade microbiana, visto que a infecção secundária, pode ser fator agravante e perpetuante da CCS.

AGRADECIMENTOS

Os autores agradecem a Pós-Graduação da UNOESTE pelo apoio financeiro.

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Tabela 1. Microrganismos isolados de secreção ocular de ambos os olhos de cães (n=65) com CCS.

Microrganismo	OD (%)	OE (%)	Total OD + OE (%)
Burkholderia cepacia	0 (0,0)	1 (1,4)	1 (0,8)
Citrobacter freundii	0 (0,0)	1 (1,4)	1 (0,8)
Corynebacterium sp.	0 (0,0)	1 (1,4)	1 (0,8)
Enterobacter gergoviae	1 (1,6)	0 (0,0)	1 (0,8)
Klebisiella pneumonae	0 (0,0)	1 (1,4)	1 (0,8)
Serratia spp.	0 (0,0)	1 (1,4)	1 (0,8)
Proteus mirabilis	1 (1,6)	1 (1,4)	2 (1,5)
Pseudomonas aeruginosa	2 (3,2)	1 (1,4)	3 (2,3)
Enterobacter aerogenes	2 (3,2)	2 (2,9)	4 (3,0)
Escherichia coli	6 (9,5)	8 (11,6)	14 (10,6)
Streptococcus sp.	7 (11,1)	8 (11,6)	15 (11,4)
Staphylococcus pseudintermedius	44 (69,8)	44 (63,8)	88 (66,7)
Total	63 (100)	69 (100)	132 (100)

OD = olho direito; OE = olho esquerdo.

Tabela 2. Sensibilidade do microrganismo frente aos antibióticos testados pelo método de Disco-difusão.

Microrganismo	Sensibilidade									
	CFZ	CIP	CLO	GEN	NEO	NOR	OFX	POL B	TET	TOB
	N=60	N=132	N=132	N=132	N=132	N=132	N=132	N=72	N=132	N=132
Burkolderia cepacia	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	1/1 (100%)	1/1 (100%)
Citrobacter freundii	1/1 (100%)	1/1 (100%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	0/1 (0%)	1/1 (100%)
Corynebacterium sp.	1/1 (100%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	0/1 (0%)	0/1 (0%)	NT	0/1 (0%)	1/1 (100%)
Enterobacter aerogenes	2/2 (100%)	2/4 (50%)	4/4 (100%)	0/4 (0%)	0/4 (0%)	2/4 (50%)	2/4 (50%)	4/4 (100%)	0/4 (0%)	2/4 (50%)
Enterobacter gergoviae	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	1/1 (100%)	1/1 (100%)
Escherichia coli	2/4 (50%)	6/14 (42,9%)	11/14(78,6%)	9/14 (64,3%)	6/14 (42,9%)	11/14 (78,6%)	13/14 (92,9%)	9/9 (100%)	8/14 (57,1%)	10/14 (71,4%)
Klebisiella pneumonae	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	0/1 (0%)	NT	1/1 (100%)	1/1 (100%)
Proteus mirabilis	2/2 (100%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	2/2 (100%)	NT	0/2 (0%)	2/2 (100%)
Pseudomonas aeruginosa	3/3 (100%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	3/3 (100%)	NT	0/3 (0%)	3/3 (100%)
Serratia sp.	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	1/1 (100%)	1/1 (100%)
Staphylococcus pseudintermedius	20/31 (64,5%)	55/88 (62,5%)	80/88 (90,9%)	70/88 (79,5%)	56/88 (63,6%)	61/88 (69,3%)	58/88 (65,9)	57/58 (98,3%)	34/88 (38,6%)	76/88 (86,4%)
Streptococcus sp.	8/12 (66,7%)	8/15 (53,3%)	7/15 (46,7%)	14/15 (93,3%)	5/15 (33,3%)	13/15 (86,7%)	14/15 (93,3%)	1/1 (100%)	7/15 (46,7%)	10/15 (66,7%)
Total	43/60 (71,6%)	81/132(61,3%)	108/132(81,8%)	104/132(78,8%)	74/132(56,1%)	97/132(73,5%)	96/132(72,7%)	71/72(98,6%)	53/132(40,2%)	109/132(82,6%)

CFZ - Cefazolina; CIP - Ciprofloxacina; CLO - Cloranfenicol; GEN - Gentamicina; NEO - Neomicina; NOR - Norfloxacina; OFX - Ofloxacina;

POL B - Polimixina B; TET - Tetraciclina; TOB – Tobramicina; NT – Não testado.

Tabela 3. Sensibilidade do microrganismo frente aos antibióticos testados pelo método E-test.

Concentração µg/mL	Antimicrobiano							
	S. pseudintermedius (N=15)				Gram Negativos (N=15)			
	CLO	MOX	OFX	TOB	CLO	MOX	OFX	TOB
0.016	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	0/30 (0%)
0.047	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	0/30 (0%)
≤0.064	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	4/30 (13,3%)	1/30 (3,3%)	0/30 (0%)
0.094	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	1/30 (3,3%)	2/30 (6,6%)	0/30 (0%)
0.125	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	4/30 (13,3%)	3/30 (10%)	0/30 (0%)
0.19	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	2/30 (6,6%)	4/30 (13,3%)	0/30 (0%)
0.25	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	2/30 (6,6%)	1/30 (3,3%)
0.38	0/30 (0%)	0/30 (0%)	2/30 (6,6%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	4/30 (13,3%)
0.50	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	6/30 (20%)
0.75	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	3/30 (10%)
1.0	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	1/30 (3,3%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)
1.5	2/30 (6,6%)	2/30 (6,6%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
2	3/30 (10%)	3/30 (10%)	1/30 (3,3%)	2/30 (6,6%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
3	4/30 (13,3%)	4/30 (13,3%)	0/30 (0%)	1/30 (3,3%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
4	1/30 (3,3%)	1/30 (3,3%)	0/30 (0%)	1/30 (3,3%)	2/30 (6,6%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
6	0/30 (0%)	0/30 (0%)	0/30 (0%)	4/30 (13,3%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
8	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)
12	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3,3%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
24	3/30 (10%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
≥32	2/30 (6,6%)	2/30 (6,6%)	11/30 (36,7%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
≥256	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
Total de cepas sensíveis	10/15(66,7%)	0/15(0%)	3/15(20%)	14/15(93,3%)	12/15(80%)	15/15(100%)	15/15(100%)	14/15 (93,3%)

Frequência de M.O. sensíveis

M.O. - Microrganismo; CLO - Cloranfenicol; MOX - Moxifloxacina; OFX - Ofloxacina; TOB - Tobramicina

*Critérios estabelecidos com base em concentrações séricas.

Tabela 4. Percentuais de concordância de resultados do E-test e Disco Difusão (DD), e estimativas dos coeficientes de concordância de Kappa.

Comparação	% concordância	Kappa	p	Interpretação
TOB (E-test x DD)	70,0%	0.3284	0.0263	Fraca
CLO (E-test x DD)	93,4%	0.8421	< 0.0001	Excelente
OFX (E-test x DD)	86,7%	0.7368	< 0.0001	Boa

TOB - Tobramicina; CLO - Cloranfenicol; OFX - Ofloxacina;

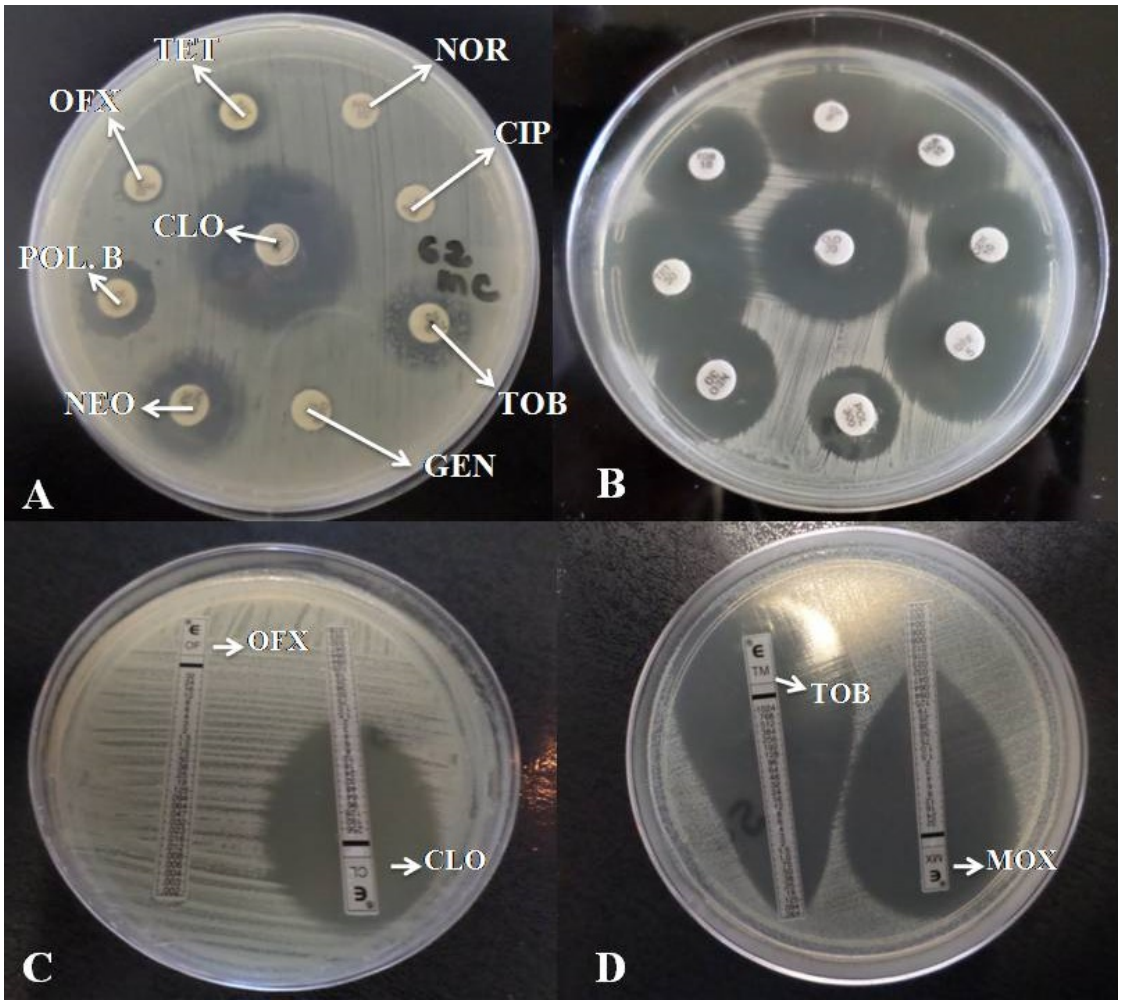

Figura 1. (A) - Perfil de sensibilidade aos antimicrobianos de um isolado de *S. pseudintermedius* pelo método disco-difusão, indicando sensibilidade ao cloranfenicol, neomicina e polimixina B. (B) - Perfil de sensibilidade aos antimicrobianos de um isolado de *S. pseudintermedius* pelo método disco-difusão, indicando sensibilidade a todos antimicrobianos testados. (C) - Perfil de sensibilidade aos antimicrobianos ofloxacina e cloranfenicol de um isolado de *S. pseudintermedius* pelo método E-test, indicando a sensibilidade de cloranfenicol. (D) - Perfil de sensibilidade aos antimicrobianos moxifloxacina e tobramicina de um isolado de *S. pseudintermedius* pelo método E-test, indicando sensibilidade de ambos.

CIP - Ciprofloxacina; CLO - Cloranfenicol; GEN - Gentamicina; NEO - Neomicina; NOR - Norfloxacina; OFX - Ofloxacina; POL. B - Polimixina B; TET - Tetraciclina; TOB - Tobramicina.

Figura 2. Sinais clínicos (secreção mucopurulenta, opacidade de córnea e neovascularização) e TLS dos animais que apresentaram resistência microbiana e tratados topicamente com antibióticos sensíveis, além do tratamento convencional para CCS com imunossupressor e lubrificante ocular. (A) OD animal N4 que apresentou sensibilidade somente à cefazolina e TLS 0 mm/min. (B) OD do mesmo animal, com melhora dos sinais clínicos e TLS 24 mm/min, após 1 mês de tratamento com colírio manipulado de cefazolina 5%, 1 gota 4x/dia. (C) OE animal N5 que apresentou sensibilidade somente à vancomicina e TLS de 5mm/min. (D) OE do mesmo animal, com melhora dos sinais clínicos e TLS 10 mm/min, após 1 mês de tratamento com colírio manipulado de vancomicina 5%, 1 gota 4x/dia. (E) OE do animal N13 que apresentou sensibilidade à polimixina B e amicacina e TLS 0 mm/min. (F) OE do mesmo animal, com melhora dos sinais clínicos e TLS 17 mm/min, após 1 mês de tratamento com colírio manipulado de polimixina B 5%, 1 gota 4x/dia.

TLS: Teste Lacrimal de Schirmer, OD: olho direito, OE: olho esquerdo

Microbial sensitivity profile of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca

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Running Title: Microbial sensitivity profile bacteria isolated dogs KCS

ABSTRACT

Objective Evaluate the microbial sensitivity profile of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca (KCS).

Procedure We evaluated 65 dogs diagnosed with KCS and 30 healthy dogs (control group). After a diagnosis of KCS, conjunctival swabs were collected, and microbiological examinations were performed, including aerobic culture, antibiogram and minimum inhibitory concentration (MIC) for chloramphenicol, tobramycin, ofloxacin and moxifloxacin.

Results Microorganisms showed the highest sensitivity by percentage to polymyxin B, tobramycin and chloramphenicol, and the lowest sensitivity to tetracycline. To determine the MIC, the fifteen most resistant strains of *Staphylococcus pseudintermedius* and the fifteen most resistant strains of Gram-negative bacteria were selected. For *S. pseudintermedius,* tobramycin demonstrated the highest percentage sensitivity, ofloxacin and moxifloxacin demonstrated the lowest sensitivity. For Gram-negative bacteria, ofloxacin, moxifloxacin, tobramycin and chloramphenicol presented excellent sensitivity. Three multi-drug resistant strains of *S. pseudintermedius* were detected. One displayed isolated sensitivity to cefazolin, another to vancomycin and another to polymyxin B and amikacin. Remission from bacterial infection was achieved after 15 days in 100% of the animals topically treated with antibiotics selected according to sensitivity.

Conclusion The species of bacteria isolated from the eyes of dogs with KCS presented variable sensitivity to the tested antibiotics. There is evidence of the emergence of quinolone-resistant strains of *S. pseudintermedius,* with further evidence of higher ocular prevalence. This reinforces the need to identify the bacteria involved and their antimicrobial susceptibility profile, as secondary infections can be exacerbating and perpetuating factors of KCS.

Key-words antibiogram, bacteria, disk-diffusion, dry eye, dogs, minimum inhibitory concentration

INTRODUCTION

Keratoconjunctivitis sicca (KCS), or dry eye, is a common ophthalmopathy which usually occurs in dogs because of a deficiency in the aqueous component of the pre-corneal tear film or because of modified quality and/or reduction in tear stability. KCS has multiple etiologies, the most prevalent being immunomediated. [1-5]

Topical therapy for KCS generally consists of immunosuppressants, such as cyclosporine, tacrolimus or pimecrolimus, and lachrymal substitutes, combined with antibiotics for treatment of secondary infections. Typical antibiotics include gentamicin, tobramycin, chloramphenicol, ofloxacin, moxifloxacin, and gatifloxacin. In addition, topical anti-inflammatories, mucolytics and parasympathomimetic stimulants of lacrimal secretion may be used. [2,5]

The ocular surface is colonized by saprophytic bacteria that, together with humoral factors, inhibit the establishment of pathogenic microorganisms. In healthy dogs, Gram-positive bacteria are principally present on the ocular surface. Species profiles vary according to geographical location, tear secretion cultivation technique, contact among other animals, population density and season.[6-8]

In eyes that have suffered an external injury, superficial inflammation of the cornea and conjunctiva are triggered, leading to a decrease in antibacterial enzymes (lactoferrin, lysozyme and peroxidase). This loss of epithelial integrity, depending on the degree and intensity of trauma and tear film modifications, favors the colonization of ocular structures by opportunistic bacteria and fungi.[7] ,

Microorganisms with high prevalence in the dogs studied include the following species: *Staphylococcus epidermidis, S. pseudintermedius, S. aureus, Streptococcus pneumoniae, Escherichia. coli, Streptococcus spp, Enterobacter* sp., and *Pseudomonas* sp. In dogs with KCS, these agents often express resistance to several of the antimicrobials principally used.

The identification of multi-drug resistance reinforces the importance of the rational use of antimicrobials, in accordance with microbial culture and susceptibility testing, to guide the choice of therapy.[8-10]

Antimicrobial therapy for eye diseases, especially conjunctivitis, keratitis and corneal ulcers, is typically performed by the use of topical antibiotic eye drops. The drops most commonly used in veterinary medicine include either chloramphenicol, aminoglycosides (neomycin, gentamicin, tobramycin), tetracyclines, and/or second-generation (ofloxacin, ciprofloxacin) and fourth-generation quinolones (gatifloxacin and moxifloxacin). [5,9]

Antimicrobial choice should be guided by sensitivity testing. The disk-diffusion method, described by Kirby-Bauer in 1966, is an easy to use, low-cost technique and is available in many diagnostic laboratories. Alternatively, the determination of the minimum inhibitory concentration (MIC), the lowest dilution of a drug capable of inhibiting the growth of microorganisms, has gained prominence due to good sensitivity, ease of implementation and high reproducibility, and resultant high reliability of results. [10]

There are few studies on the sensitivity profiles of bacterial agents involved in secondary eye infections in dogs with KCS. Therefore, the aim of this study was to evaluate antimicrobial sensitivity through the disk-diffusion method and determination of MIC of bacteria isolated from the ocular secretions of dogs with KCS.

MATERIALS AND METHODS

The study was conducted at the Veterinary Hospital of the UNOESTE, Presidente Prudente, SP. We evaluated 65 dogs diagnosed with KCS, with no predilection for race, age or gender. Dogs were registered under an authorization form (Term of Free and Informed Consent - TFIC) signed by the owners and those responsible for the project. The control group consisted of 30 dogs from the kennel of an educational institution. All were healthy

(evaluated through clinical and laboratory examinations) and without eye problems (evaluated through visual examination with slit lamp, the Schirmer Tear test, Fluorescein test, Tear Film Break-up Time and tonometry). The study was conducted and approved in accordance with the animal testing regulations of the Ethics Committee on Animal Use (CEUA) of UNOESTE (Protocol N. 1802 and 1803) and is in accordance with the rules of the ARVO (Association for Research in Vision and Ophthalmology - Statement for the use of animals in ophthalmic and visual research).

The diagnosis of KCS was based on clinical signs and specific ophthalmic tests that included the Schirmer Tear test (STT) and Tear Film Break-up Time (TBUT) or Break Up Time (BUT). To perform the STT, a 0.5 cm filter paper strip (Teste de Schirmer Ophthalmos® ,São Paulo, Brazil) was introduced into the conjunctival sac for a period of 1 minute. The result was considered positive for dogs who presented values ≤ 10 mm/min. For the TBUT test, a sterile strip of fluorescein 1% (Ophthalmos Laboratory, São Paulo, Brazil) soaked in 1 drop of saline solution was placed in contact with the conjunctival sac. After the dog blinked twice, the eyelid was immobilized, and the cornea was observed with a biomicroscope (portable slit lamp) to determine the TBUT. Two successive tests were performed to calculate the mean TBUT value. Mean TBUT values of ≤ 10 sec were considered positive for KCS.[11]

To determine the existence of corneal ulcers, the fluorescein test (FT) was used, which consisted of a 1% sterile fluorescein strip (Ophthalmos Laboratory, São Paulo, Brazil) soaked in a drop of saline solution and placed against the conjunctival sac. The test was positive for the presence of ulcers when areas of the cornea exposed to light presented a greenish tint. Additionally, the Rose Bengal test (RBT) was performed using rose bengal 1% eye drops (Ophthalmos Laboratory, São Paulo, Brazil) after application of anesthetic eye drops for detection of corneal and conjunctival staining of cells devitalized by KCS. The test was

considered positive for the presence of cells devitalized by KCS when areas of the cornea or conjunctiva revealed pink staining. [11]

After the diagnosis of KCS, samples of ocular secretions were collected from the lower conjunctivae of both eyes with sterile cotton swabs. Samples were plated on Petri dishes containing 5% defibrinated bovine blood agar and MacConkey agar. The inoculated plates were incubated under aerobic conditions at 37°C for 24-48 hours. Isolated bacterial colonies were classified according to morphology, staining and biochemical characteristics.[12] Initially, the isolates for sensitivity testing were seeded in brain-heart infusion broth and incubated at 37°C for 24-48 hours under aerobic conditions. After this period, the broths were diluted in saline until reaching turbidity of 0.5 on the McFarland scale. One aliquot of the broth was then seeded superficially on Mueller-Hinton agar plates with a sterile swab.

After the 30-minute period necessary for inoculum absorption, filter paper disks (Cecon, São Paulo, Brazil) were deposited evenly over the agar surface. These disks contained the following antibacterial agents: cefazolin (30 mcg), ciprofloxacin (5 mcg), chloramphenicol (30 mcg), gentamicin (10 mcg), neomycin (30 mcg), norfloxacin (10 mcg), ofloxacin (5 mcg), polymyxin B (300 mcg), tetracycline (30 mcg), tobramycin (10 mcg) and vancomycin (30 mcg) (Fig. 1 A, B). After 18 hours of incubation, the diameter of the inhibition zones around the disks was measured with a caliper, and the results, expressed in millimeters, were compared to international standards for the interpretation of results. [12]

The minimum inhibitory concentrations of the most relevant and potentially pathogenic isolates for dogs were evaluated for sensitivity to chloramphenicol, tobramycin, ofloxacin and moxifloxacin through the E-test method (Probac, São Paulo, Brazil). One aliquot of the broth was seeded superficially on Mueller-Hinton agar plates with a sterile swab. After 30 minutes, commercial plastic strips impregnated with chloramphenicol (0.016 – 256 µg/mL), tobramycin (0.064 – 1024 µg/mL), ofloxacin and moxifloxacin (0.002 – 32 µg/mL) were

deposited on the agar surface. After incubation at 37°C for 24 hours, the observed points of growth inhibition corresponding to the minimum inhibitory concentrations were computed for each agent (Fig. 1C, D). The patterns observed were compared with the cutoffs established by the Clinical and Laboratory Standards Institute (2012)[12] to classify isolates as sensitive, partially sensitive and resistant. Cutoff points for *S. pseudintermedius* were: chloramphenicol S = ≤ 8 μg/mL; moxifloxacin = S ≤ 0.5 μg/mL; ofloxacin S = ≤ 1 μg/mL and tobramycin S = ≤ 8 μg/mL. The cutoff points for gram-negative bacteria were: chloramphenicol S = ≤ 8 μg/mL; moxifloxacin S = ≤ 0.5 μg/mL; ofloxacin S = ≤ 2 μg/mL and tobramycin S= ≤ 4 μg/mL.

The prevalence of ocular bacterial infection was determined for both the dogs with KCS and those in the control group and was estimated by point and interval with 95% confidence. The percentages of positive dogs in the KCS and control groups were compared using the binomial test. The agreement between the E-test and Disk-diffusion sensitivity test results was measured by analyzing the Kappa coefficient of agreement. A level of significance of 5% was adopted.

RESULTS

Of the 65 dogs diagnosed with KCS, 36 (55%) were female and 29 (45%) were male. The most common breeds of selected animals were Lhasa Apso and mixed breed (SRD) with 17 (26.2%) dogs each, followed by 10 Poodles (15.4%), 5 Pinschers (7.7 %), 3 Yorkshire Terriers (4.6%), 2 Pit Bulls (3.1%) and 11 of other breeds (1.5%).

In the group of animals with KCS, 59 of the 65 dogs (90.8%, CI 95% = 83.7 -97.8%) were culture-positive for at least one eye. In the control group, 11 of the 30 dogs (36.7%, CI 95% = 19.4 -53.9%) were culture-positive for at least one eye. These percentages were significantly different between the control and KCS groups ($p < 0.0001$).

All isolates from the control group were classified as S. *pseudintermedius*. For animals with KCS, the microbiological profile consisted of Gram-positive and Gram-negative bacterial species (Table 1). It was found that 7.5% of the microorganisms were isolated to only one eye, whereas 92.4% were concordant for both eyes.

The sensitivity of the isolated agents in accordance with the disk-diffusion method is described in Table 2. To determine the MIC through the E-test method, fifteen S. *pseudintermedius* and fifteen gram-negative strains (eleven of *E. coli*, three of *Enterobacter aerogenes* and one of *Citrobacter freundii*) were selected (Table 3). High agreement was verified between the disk-diffusion and E-test methods for chloramphenicol, good agreement for ofloxacin and weak agreement for tobramycin (Table 4).

Dogs with KCS and positive isolates characterizing eye infection were treated after sensitivity testing with appropriate antimicrobial eye drops. In 3 (4.6%) of the 65 dogs, *S. pseudintermedius* strains characterizing multi-resistance were detected. This was defined as resistance to nine or more principal antimicrobials, including oxacillin. In light of these results, these isolates were subjected to new sensitivity tests that revealed that one isolate (N4) was sensitive to cefazolin (Fig. 2 A, B), one (N5) to vancomycin (Fig. 2 C, D) and one (N13) to polymyxin B and amikacin (Fig. 2 E, F). Due to the absence of suitable commercial preparations in Brazil, it was decided to prescribe manipulated compounded ophthalmic solutions containing these antimicrobials were administered (Laboratório Ophthalmos®, São Paulo, Brazil). In 100% of the treated animals, remission of bacterial infection was achieved after 15 days of treatment (1 drop in both eyes, four times daily).

DISCUSSION

The high percentage of dogs with KCS with positive cultures (90.8%) compared to the control group (36.7%) reinforces the importance of identifying the profile and sensitivity

of bacterial agents to guide antibiotic treatment. [13,14] Dogs with KCS present tear film deficiencies, either in production or evaporation. Consequently, there is a reduction in antibacterial enzyme levels (lactoferrin, lysozyme and peroxidase) that favors the colonization of opportunistic bacteria in the conjunctival sac. In turn, this contributes to a higher frequency of positive cultures compared to healthy dogs, which corroborates the results observed. [2-7]

The pathogen with the highest prevalence in the eyes of the animals was *S. pseudintermedius,* isolated from approximately 67% of the investigated samples. *S. pseudintermedius* is a coagulase-positive species, saprophytic in the skin and mucous of dogs, that commonly colonizes the eyes of healthy dogs.[15-18]

A predominance of enteric Gram-negative organisms was observed in relation to other Gram negatives. *Escherichia coli* was most prevalent, (approximately 11% of isolates), followed by *Enterobacter aerogenes* (3%). These results are consistent with other studies in which *Escherichia coli* accounted for 10% of samples isolated. [18-19] *P. aeruginosa*, a microorganism capable of secreting enzymes that cause liquefaction of corneal stroma and rapid progression to ulceration, [18] was present in low frequency compared to other agents, with sensitivity to all antibiotics tested.

When assessing the overall sensitivity of isolated agents through the disk-diffusion method, tobramycin, the aminoglycoside tested, was more effective than the quinolones tested, ofloxacin and moxifloxacin. Therefore, aminoglycosides may be more suitable for empirical or prophylactic treatment of ophthalmopathies in dogs compared to quinolones, such as ciprofloxacin, moxifloxacin and gatifloxacin-antimicrobials to which the described microorganisms have developed remarkable resistance in the past decade. [18, 20,21]

Results of the present study revealed good efficacy of polymyxin B and chloramphenicol. These results agree with those reported by other authors. Chloramphenicol

is a broad spectrum antibiotic and has been more widely used since the emergence of methicillin-resistant staphylococci. However, due to its toxic effects in humans, use of the drug has been limited. [22]

Three dogs presented *S. pseudintermedius* strains resistant to oxacillin, an antimicrobial considered important as a marker of multi-drug resistance in human ocular infections. [20] Other authors have also reported increased resistance of *S. pseudintermedius* in canine ocular infections.[23-25] In the three cases of resistance observed in the present study, additional sensitivity tests revealed that these isolates were sensitive to two exclusively injectable antibiotics in Brazil, vancomycin, a glycopeptide antibiotic used in hospital infections, and cefazolin, an antibiotic of the cephalosporin group.

Tetracycline had low efficacy against these isolates, with only 40.2% sensitive to this antibiotic. This result is consistent with other studies that reported resistance to tetracycline in more than 50% of *S. pseudintermedius* strains isolated from ophthalmic diseases.[26]

Sensitivity testing of the isolated agents using the E-test method revealed that tobramycin presented good effectiveness against both Gram-negative organisms and Gram-positive organisms such as *S. pseudintermedius*. This is consistent with the results observed in other studies. [27,28] According to the E-test, the quinolones tested had low effectiveness against *S. pseudintermedius*, with resistance rates above 80%. Considering that moxifloxacin and ofloxacin have recently entered frequent clinical use for treatment of canine ophthalmopathies, the results point to the possible emergence of resistant strains among animals in the region.[26]

In contrast, Gram-negative bacteria were sensitive to the quinolones tested, which suggests that selection pressure on these microorganisms is lower than that observed for *S. pseudintermedius*. However, multi-drug resistant *E. coli* strains, commonly observed in

urinary infections, are an exception to this finding.[29,30] It was found that excellent agreement existed between the results of E-test and Disk-diffusion tests for chloramphenicol and ofloxacin, but limited agreement was observed between tests for tobramycin.

In conclusion, it was found that the bacteria species isolated from the eyes of dogs with KCS have variable sensitivity to the antibiotics tested. The emergence of quinolone-resistant strains of *S. pseudintermedius*, found in high prevalence among the eyes of these animals, reinforces the need to identify the antimicrobial susceptibility profile of the bacteria involved, as secondary infections can be an exacerbating and perpetuating factor of KCS.

ACKNOWLEDGEMENTS

The authors would like to thank the Postgraduate Program of UNOESTE for financial support.

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Table 1. Microorganisms isolated from ocular secretions of both eyes of dogs (n = 65) with KCS.

Microorganism	Right Eye (%)	Left Eye (%)	Total (%)
Burkholderia cepacia	0 (0.0)	1 (1.4)	1 (0.8)
Citrobacter freundii	0 (0.0)	1 (1.4)	1 (0.8)
Corynebacterium sp.	0 (0.0)	1 (1.4)	1 (0.8)
Enterobacter gergoviae	1 (1.6)	0 (0.0)	1 (0.8)
Klebsiella pneumoniae	0 (0.0)	1 (1.4)	1 (0.8)
Serratia spp.	0 (0.0)	1 (1.4)	1 (0.8)
Proteus mirabilis	1 (1.6)	1 (1.4)	2 (1.5)
Pseudomonas aeruginosa	2 (3.2)	1 (1.4)	3 (2.3)
Enterobacter aerogenes	2 (3.2)	2 (2.9)	4 (3.0)
Escherichia coli	6 (9.5)	8 (11.6)	14 (10.6)
Streptococcus sp.	7 (11.1)	8 (11.6)	15 (11.4)
Staphylococcus pseudintermedius	44 (69.8)	44 (63.8)	88 (66.7)
Total	63 (100)	69 (100)	132 (100)

Table 2. Sensitivity of microorganisms to the antibiotics tested through the disk-diffusion method.

Microorganism	**Sensitivity**									
	CFZ	CIP	CLO	GEN	NEO	NOR	OFX	POL B	TET	TOB
	N=60	N=132	N=132	N=132	N=132	N=132	N=132	N=72	N=132	N=132
Burkholderia cepacia	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	1/1 (100%)	1/1 (100%)
Citrobacter freundii	1/1 (100%)	1/1 (100%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	0/1 (0%)	1/1 (100%)
Corynebacterium sp.	1/1 (100%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	0/1 (0%)	0/1 (0%)	NT	0/1 (0%)	1/1 (100%)
Enterobacter aerogenes	2/2 (100%)	2/4 (50%)	4/4 (100%)	0/4 (0%)	0/4 (0%)	2/4 (50%)	2/4 (50%)	4/4 (100%)	0/4 (0%)	2/4 (50%)
Enterobacter gergoviae	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	1/1 (100%)	1/1 (100%)
Escherichia coli	2/4 (50%)	6/14 (42.9%)	11/14(78.6%)	9/14 (64.3%)	6/14 (42.9%)	11/14 (78.6%)	13/14 (92.9%)	9/9 (100%)	8/14 (57.1%)	10/14 (71.4%)
Klebsiella pneumoniae	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	0/1 (0%)	NT	1/1 (100%)	1/1 (100%)
Proteus mirabilis	2/2 (100%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	2/2 (100%)	NT	0/2 (0%)	2/2 (100%)
Pseudomonas aeruginosa	3/3 (100%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	3/3 (100%)	NT	0/3 (0%)	3/3 (100%)
Serratia sp.	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	NT	1/1 (100%)	1/1 (100%)
Staphylococcus pseudintermedius	20/31 (64.5%)	55/88 (62.5%)	80/88 (90.9%)	70/88 (79.5%)	56/88 (63.6%)	61/88 (69.3%)	58/88 (65.9)	57/58 (98.3%)	34/88 (38.6%)	76/88 (86.4%)
Streptococcus sp.	8/12 (66.7%)	8/15 (53.3%)	7/15 (46.7%)	14/15 (93.3%)	5/15 (33.3%)	13/15 (86.7%)	14/15 (93.3%)	1/1 (100%)	7/15 (46.7%)	10/15 (66.7%)
Total	43/60 (71.6%)	81/132(61.3%)	108/132(81.8%)	104/132(78.8%)	74/132(56.1%)	97/132(73.5%)	96/132(72.7%)	71/72(98.6%)	53/132(40.2%)	109/132(82.6%)

CFZ - Cefazolin; CIP - Ciprofloxacin; CLO - Chloramphenicol; GEN - Gentamicin; NEO - Neomycin; NOR - Norfloxacin; NT - Not Tested; OFX - Ofloxacin; POL B - Polymyxin B; TET - Tetracycline; TOB - Tobramycin.

Table 3. Sensitivity of microorganisms to the antibiotics tested through the E-test method.

Concentration µg/mL	Antimicrobial							
	S. pseudintermedius (N=15)				Gram Negatives (N=15)			
	CLO	MOX	OFX	TOB	CLO	MOX	OFX	TOB
0.016	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	0/30 (0%)
0.047	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	0/30 (0%)
≤0.064	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	4/30 (13.3%)	1/30 (3.3%)	0/30 (0%)
0.094	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	1/30 (3.3%)	2/30 (6.6%)	0/30 (0%)
0.125	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	4/30 (13.3%)	3/30 (10%)	0/30 (0%)
0.19	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	2/30 (6.6%)	4/30 (13.3%)	0/30 (0%)
0.25	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	2/30 (6.6%)	1/30 (3.3%)
0.38	0/30 (0%)	0/30 (0%)	2/30 (6.6%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	4/30 (13.3%)
0.50	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	6/30 (20%)
0.75	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	3/30 (10%)
1.0	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	1/30 (3.3%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)
1.5	2/30 (6.6%)	2/30 (6.6%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
2	3/30 (10%)	3/30 (10%)	1/30 (3.3%)	2/30 (6.6%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
3	4/30 (13.3%)	4/30 (13.3%)	0/30 (0%)	1/30 (3.3%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
4	1/30 (3.3%)	1/30 (3.3%)	0/30 (0%)	1/30 (3.3%)	2/30 (6.6%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
6	0/30 (0%)	0/30 (0%)	0/30 (0%)	4/30 (13.3%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
8	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)
12	0/30 (0%)	0/30 (0%)	0/30 (0%)	1/30 (3.3%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
24	3/30 (10%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
≥32	2/30 (6.6%)	2/30 (6.6%)	11/30 (36.7%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
≥256	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	3/30 (10%)	0/30 (0%)	0/30 (0%)	0/30 (0%)
Total of sensitive strains	10/15(66.7%)	0/15(0%)	3/15(20%)	14/15(93.3%)	12/15(80%)	15/15(100%)	15/15(100%)	14/15 (93.3%)

Frequency of sensitive M.O.s. M.O. - Microorganism; CLO - Chloramphenicol; MOX - Moxifloxacin; OFX - Ofloxacin; TOB - Tobramycin

* Criteria established based on serum concentrations.

Table 4. Percentage agreement of results from E-test and Disk-diffusion (DD) tests, and estimates of the Kappa coefficients of agreement.

Comparison	% agreement	Kappa	p	Interpretation
TOB (E-test x DD)	70.0%	0.3284	0.0263	Weak
CLO (E-test x DD)	93.4%	0.8421	< 0.0001	Excellent
OFX (E-test x DD)	86.7%	0.7368	< 0.0001	Good

TOB - Tobramycin; CLO - Chloramphenicol; OFX - Ofloxacin

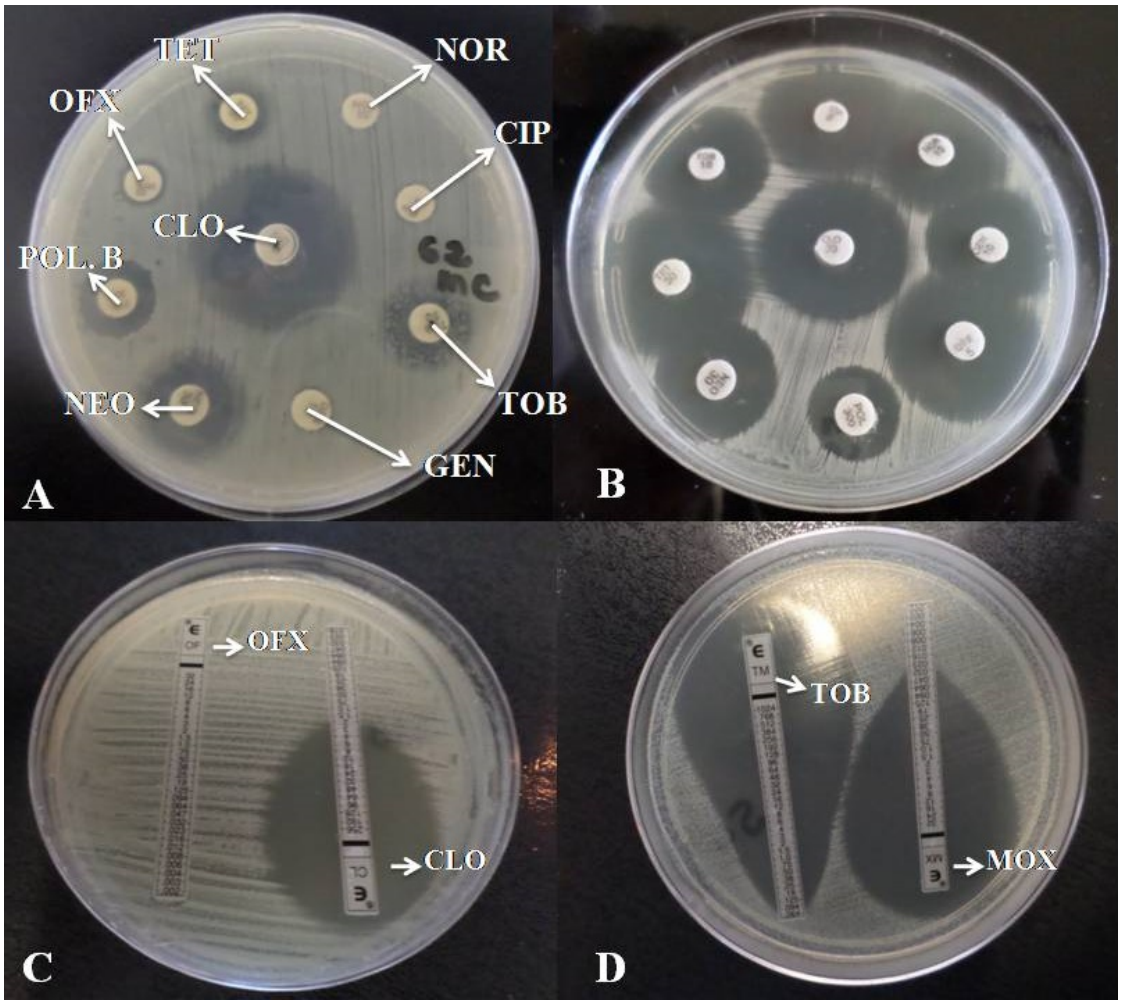

Figure 1. (A) - Sensitivity profile to antimicrobials of an isolate of *S. pseudintermedius* through the Disk-diffusion method, indicating sensitivity to chloramphenicol, neomycin and polymyxin B. (B) - Sensitivity profile to antimicrobials of one isolate of *S. pseudintermedius* through the Disk-diffusion method, showing sensitivity to all antimicrobials tested. (C) - Sensitivity profile to the antimicrobials ofloxacin and chloramphenicol of one isolate of *S. pseudintermedius* through the E-test method, indicating sensitivity to chloramphenicol. (D) - Sensitivity profile to the antimicrobials moxifloxacin and tobramycin of one isolate of *S. pseudintermedius* through the E-test method, indicating sensitivity to both.

CIP - Ciprofloxacin; CLO - Chloramphenicol; GEN - Gentamicin; NEO - Neomycin; NOR - Norfloxacin; OFX - Ofloxacin; POL. B - Polymyxin B; TET - Tetracycline; TOB - Tobramycin.

Figure 2. Clinical signs (purulent secretion, corneal opacity and neovascularization) and STT of animals that presented microbial resistance, topically treated with sensitive antibiotics, in addition to conventional treatment for KCS with an immunosuppressant and ocular lubricant. (A) OD of animal N4 which presented sensitivity only to cefazolin and STT 0 mm/min. (B) OD of the same animal, with improvement in clinical signs and STT 24 mm/min after 1 month of treatment with manipulated cefazolin 5% eye drops, 1 drop 4x/day. (C) OS of animal N5 which presented sensitivity only to vancomycin and STT 5 mm/min. (D) OS of the same animal, with improvement in clinical signs and STT 10 mm/min, after 1 month of treatment with manipulated vancomycin 5% eye drops, 1 drop 4x/day. (E) OS of animal N13 which presented sensitivity to polymyxin B and amikacin and STT 0 mm/min. (F) OS of the same animal, with improvement in clinical signs and STT 17 mm/min, after 1 month of treatment with manipulated polymyxin B 5% eye drops, 1 drop 4x/day.

STT: Schirmer Tear Test, OD: *oculus dexter* - right eye, OS: *oculus sinister* - left eye

ANEXO
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Edited By: David A. Wilkie, The Ohio State University

Impact Factor: 1.062

ISI Journal Citation Reports © Ranking: 2014: 57/133 (Veterinary Sciences)

Online ISSN: 1463-5224

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