UNIVERSIDADE FEDERAL DO PARANÁ

THIAGO ALEGRE COELHO FERREIRA

CONTRIBUIÇÕES PARA A FARMACOLOGIA DIAGNÓSTICA NA OFTALMOLOGIA VETERINÁRIA

CURITIBA

2019

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Tese apresentada ao Programa de Pós-graduação em Ciências Veterinárias, do Setor de Ciências Agrárias, da Universidade Federal do Paraná, como requisito parcial para obtenção do título de Doutor em Ciências Veterinárias.

Orientador: Professor Dr. Fabiano Montiani-Ferreira

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RESUMO

A farmacologia diagnóstica é um importante nicho de pesquisa dentro da oftalmologia veterinária, tamanha sua importância e impacto de suas descobertas. A gama de possibilidades e de drogas disponíveis aumenta na mesma velocidade em que as pesquisas avançam rumo à precisão da localização do foco da doença. Para isso, a compreensão dos diferentes mecanismos de ação, bem como limitações e possíveis alterações no organismo ou do meio que o cerca, são de suma importância para evitar distorções na interpretação dos resultados. No primeiro capítulo aborda-se uma modalidade farmacológica relativamente nova no que diz respeito ao diagnóstico, principalmente na oftalmologia veterinária. Trata-se de uma ideia de aplicação ocular de uma solução de microbolhas que já vinha sendo empregada como contraste na ultrassonografia de outros órgãos ou no próprio bulbo ocular mas com outras finalidades. Nesta proposta foram abordadas diferentes espécies de aves, as quais receberam uma injeção intravenosa da solução de microbolhas para evidenciar o *pecten*, uma iniciativa até então inédita no meio científico. Constatou-se que o *pecten* foi evidenciado pelo contraste sem extravasamento deste para o corpo vítreo e sem efeitos colaterais para as aves na dose utilizada. No segundo capítulo, os principais corantes vitais utilizados na oftalmologia médica e veterinária foram testados para sua capacidade de inibição ou permissibilidade do crescimento de determinadas espécies bacterianas. Testes de Kirk-Bauer e de eficácia antimicrobiana foram realizados a fim de verificar as capacidades citadas anteriormente em diferentes formulações para os mesmos corantes, ora em *strips*, ora em soluções, neste caso com ou sem conservantes. Os resultados mostraram que cuidados devem ser tomados principalmente com fluoresceína sem conservante para evitar o crescimento de *Escherichia coli* e *Pseudomonas aeruginosa*, sendo aconselhável não utilizar este corante nesta condição. Concluiu-se também que coletas para cultura e antibiograma devem ser realizadas antes de se corar a superfície ocular devido ao potencial antimicrobiano de alguns corantes

Palavras-chave: microbolhas, aves, *pecten*, corantes vitais, fluoresceína, *Pseudomonas aeruginosa*, oftalmologia, veterinária.

ABSTRACT

Diagnostic pharmacology is an important research niche within veterinary ophthalmology due to its importance and impact of its findings. The range of possibilities and drugs available increases at the same rate as research advances toward the precise location of the disease's focus. For this, the understanding of the different mechanisms of action, as well as limitations and possible alterations in the organism or the surrounding environment, are extremely important to avoid distortions in the interpretation of the results. The first chapter covers a relatively new pharmacological modality regarding diagnosis, especially in veterinary ophthalmology. It is an idea of ocular application of a microbubble solution that has already being used as a contrast in the ultrasound of other organs or in the eye bulb itself but for other purposes. Different species of birds, which received an intravenous injection of the microbubbles solution to highlight the pecten were approached in this proposal, an initiative previously unheard of in the scientific environment. It was found that pecten was evidenced without extravasation by the contrast from it to the vitreous body and without side effects to the birds at the dose used. In the second chapter, the main vital dyes used in medical and veterinary ophthalmology were tested for their ability to inhibit or allow the growth of certain bacterial species. Kirk-Bauer and antimicrobial efficacy tests were performed to verify the aforementioned capabilities in different formulations for the same dyes, time in strips, time in solutions, in this case with or without preservatives. The results showed that care should be taken mainly with preservative-free fluorescein to prevent the growth of *Escherichia coli* and *Pseudomonas aeruginosa*. It is advisable not to use this dye in this condition. It was also concluded that sample collection for culture and sensitivity should be performed before staining the ocular surface due to the antimicrobial potential of some dyes.

Key words: microbubbles, birds, pecten, vital dyes, fluorescein, *Pseudomonas aeruginosa*, ophthalmology, veterinary.

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SUMÁRIO

1. INTRODUÇÃO

A farmacologia diagnóstica é um campo da farmacologia que abrange os fármacos que, de alguma forma, contribuem para elucidar processos patológicos que acometem determinado órgão ou tecido. A lista de grupos farmacológicos que a compõe é extensa e concerne desde corantes vitais, neuro-estimuladores e neuro-inibidores, até contrastes, esta última classe, mais extensa que as outras, abrangendo desde químicos comuns como sulfato de bário como contraste para o aparelho gastrintestinal, até metaiodobenzilguanidina, marcada com ^{123}Iodo ou ^{131}Iodo para cintilografia, ou mesmo o gadolinium para imagens moleculares (Coyne, 2006; Simal, 2011). Na oftalmologia veterinária a farmacologia diagnóstica não é menos importante. Pode-se destacar principalmente o emprego de corantes vitais que desempenham diferentes funções, desde a localização de lesões, como é o caso da fluoresceína nas úlceras de córnea, até a evidenciação de estruturas durante o transoperatório, como é o caso do azul de tripano evidenciando a cápsula anterior da lente na facoemulsificação (Gellat et al., 2013). A respeito dos corantes vitais que evidenciam lesões na superfície ocular, muito se discutiu ao longo das últimas décadas sobre o potencial de permissibilidade para o crescimento bacteriano, principalmente a fluoresceína, a qual sempre esteve associada ao crescimento de *Pseudomonas aeruginosa* (Theodore, 1952; Cello, 1958; Brown, 1968). Sobre essa associação foram publicados trabalhos utilizando diferentes metodologias e sempre concluindo que cuidados deveriam ser tomados na utilização da fluoresceína, alguns inclusive desaconselhando sua utilização (Theodore, 1952; Cello, 1958; Brown, 1968; Claoué, 1986). Apesar disso, nenhum trabalho cientifico publicado abordou de forma objetiva como diferentes bactérias, incluindo a *Pseudomonas aeruginosa*, se comportariam diante da formulação em solução sem conservante em comparação com a formulação com conservante, o mesmo serviria para os outros corantes vitais mais utilizados na rotina clínica, como o verde de lissamina e o rosa de bengala, sendo que apenas um trabalho apresentado em congresso internacional avaliou algo parecido, mas apenas com corantes sem conservantes (Malta et al., 2006). Outros trabalhos também analisaram qual seria o potencial antibacteriano desses mesmos corantes em forma de *strips*, ou fitas estéreis, mas sem comparar uma ampla variedade de bactérias em relação à diferentes concentrações dos corantes (Roy et al., 1998).

Ainda embrionário dentro da oftalmologia veterinária, o uso de contrastes de microbolhas para evidenciar estruturas oculares na ultrassonografia vem ganhando espaço dentro das pesquisas, primeiramente com animais de laboratório (Torres et al., 2017; Kang et al., 2013; Yuan et al., 2010). A passos curtos, mais pesquisas têm surgido empregando cães como foco, com o intuito de padronizar e validar este tipo de exame e torná-lo aplicável à rotina clínica (Hong et al., 2019). Até a publicação do recente Capítulo 1 desta tese, em meados de 2018, nenhuma outra pesquisa empregando o uso de microbolhas havia tido como foco olhos de aves. Por haver características próprias nos olhos desta classe zoológica, decidimos realizar a pesquisa tendo como foco a estrutura mais intrigante dentro do aparelho visual das aves, o *pecten*. Um desafio inicial da pesquisa foi em achar uma dose segura, uma vez que nenhuma pesquisa até então havia testado o contraste em aves. Um estudo piloto foi realizado e uma vez definida a dose segura (4,5 µg/Kg) que evidenciasse o *pecten*, se iniciou o experimento. Diferentes espécies de aves sem sinais de doenças oculares ou sistêmicas foram selecionadas do Instituto Ambiental do Paraná e algumas da Fazenda Canguiri da Universidade Federal do Paraná. As aves foram sedadas, foram introduzidos cateteres intravenosos em suas veias e receberam uma dose intravenosa do contraste de microbolhas (hexafluoreto de enxofre). Após alguns segundos, pôde-se observar o *pecten* que foi evidenciado sobremaneira pelo fluxo das microbolhas em seus capilares. Análise de textura antes e depois do contraste foi utilizada para aferir a diferença entre os níveis de cinza de uma região de interesse (*pecten*) de maneira objetiva e quantitativa e realizar a análise estatística. Houve diferença estatística entre os níveis de cinza nas regiões de interesse analisadas, indicando que o uso do contraste de microbolhas a base de hexafluoreto de enxofre é eficaz para evidenciar o *pecten* na ultrassonografia ocular em aves.

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CAPÍTULO I

The use of sulfur hexafluoride microbubbles for contrast-enhanced ocular ultrasonography of the *pecten oculi* in birds

The use of sulfur hexafluoride microbubbles for contrast-enhanced ocular ultrasonography of the *pecten oculi* in birds

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Abstract

The *pecten oculi* is a vascular and pigmented structure localized within the posterior segment of all avian eyes. Its primary function is not fully understood yet. *Objective*: Since ultrasonography (US) is a useful imaging modality for evaluation of the *pecten oculi*, the objective of this study was to investigate the utility of an intravenous contrast solution of sulfur hexafluoride (SF6) microbubbles as a means of enhancing visualization of the *pecten oculi* in normal birds. *Animals studied*: Ten adult individuals of the following avian species were evaluated: 1 roadside hawk (*Rupornis magnirostris*), 1 stygian owl (*Asio stygius*), 2 striped owls (*Asio clamator*), 2 burrowing owls (*Athene cunicularia*), 2 ring-neck parakeets (*Psittacula krameri*), and 2 domestic chickens (*Gallus gallus domesticus*). *Procedure(s)*: After baseline ocular sonograms were obtained in sedated animals, 4.5 µg/kg of a contrast solution containing SF6 microbubbles was administered intravenously and US of the right eye was immediately performed. US was continued during injection in order to provide real time imaging of the *pecten oculi* during vascular perfusion of contrast material. *Results*: Within 2-3 seconds following intravenous contrast administration, microbubbles reached the *pecten oculi* of all birds investigated and provided significant ultrasonographic contrast enhancement. *Conclusions*: SF6 microbubble contrast ultrasonography in birds is a safe and easy procedure that provides increased contrast and enhanced visualization of the *pecten oculi*. Future use may enable further discovery of its physiologic functions and aid in the development of therapeutic plans for avian intraocular disease.

Key words: ophthalmology, avian, eye, retina, diagnostic imaging

1 INTRODUCTION

The avian eye has several distinctive anatomic and physiologic features when compared to the eyes of other vertebrate taxa.[1] A large variability in eye shape, mechanisms of both lenticular and corneal accommodation, variable numbers and positions of retinal specializations, and the presence of the *pecten oculi* are among the most conspicuous features.[1-5] The pecten oculi is of particular interest as there is currently no consensus for its most important physiologic role.[6-8] It is a pigmented and highly vascularized intraocular structure projecting anteriorly from the optic disc into the vitreous body.[1,9-11] Three different morphologies have been described: 1) conical (e.g. as found in kiwis); 2) vaned (e.g. as found in ostriches, rheas, and tinamous), or 3) pleated (e.g. as found in all the other avian species).[12-16] A number of hypotheses have been reported as to the function of the *pecten oculi*, including nutritional support for the anangiotic (without blood vessels) retina,[17] regulation and maintenance of intraocular pH and pressure,[9,18] physical stabilization of the vitreous body,[11] reduction of intraocular glare,[19] maintenance of the blood ocular barrier for the retina and vitreous body,[19,20] and aqueous humor production.[7,8,21] However, the precise role of the *pecten oculi* in ophthalmic diseases also has not been fully described.

Structural detail of the pecten oculi has been elucidated in anatomical studies using scanning electron and light microscopy.[10,15,22,23] However, advanced diagnostic imaging investigations (i.e. optical coherence tomography – OCT) may better help to understand the precise role of the *pecten oculi* in normal ocular health and in disease pathogenesis.[24-27] Ultrasonography (US) is a widely available, quick, and noninvasive imaging technique with multiple modalities (e.g. color and pulsed Doppler ultrasonography; D-US), that has been successfully used to investigate the *pecten oculi*.[7,8,28] Physiologic parameters for resistive and pulsatility indexes of the pectinate artery have been investigated using D-US in both American pekin ducks (*Anas platyrhynchos domestica*) and harpy eagles (*Harpia harpyja*). [7,8] However, it is still unclear whether arterial hypotension or hypertension could be diagnosed by D-US of *pecten oculi* vessels in different ocular diseases. Despite the availability, utility, and multiple modalities of ultrasonography for visualization of the *pecten oculi*, its moderate echogenicity and often indistinct structure on ultrasound can limit precise visualization making the diagnosis of potential abnormalities difficult.

Real-time, contrast-enhanced ultrasonography (CE-US) uses intravenously administered contrast media that contains gas-filled and stabilized microbubbles, such as sulfur hexafluoride (SF6), that typically measure 5-10 µm in diameter. The small size of the bubbles makes it possible to reach the microvasculature of various organ systems. In particular, SF6 is a poorly soluble gas that has been shown to be safe in a variety of clinical settings including pediatric medicine.[29] A marked difference in echogenicity between the gas inside the microbubbles and the surrounding soft tissue facilitates imaging of blood perfusion in different organs.[30-32] CE-US has been successfully employed to investigate the vascular and microvascular nature of the liver, pancreas, spleen, heart, brain, kidneys, and eye/orbit in both humans and various animals as a means to better depict lesions or monitor tumor growth.[30-37] The liver, spleen, and pancreas were the organs first targeted for studies on the efficacy of microbubble contrast, but others uses of CE-US have since been described, including evaluation of atherosclerosis and intraoperative visualization of a brain tumors.[30-34] In veterinary medicine, microbubble CE-US has been used in the evaluation of normal kidney structure in cats,[35] the washout ratio in the hepatic vein in dogs,[38] and in the diagnosis of non-cardiac thoracic disorders of dogs and cats.[39] To the authors´ knowledge, there are no previous reports regarding the use of CE-US to investigate the *pecten oculi* in normal birds

Several reports of ophthalmic applications of CE-US are available. Detection of intraocular or orbital space occupying lesions has shown to be a valuable application of CE-US, where microbubble contrast can enable successful detection of blood flow through the small diameter of intraocular vessels.[36,40,41] For example, one study showed a correlation between CE-US and histologic results of assessing microvascular density and blood volume in murine uveal melanomas, which suggests that CE-US is a reliable exam modality for evaluation of intraocular tumors even in small species.[37] Similar results were shown more recently in a study evaluating CE-US and histologic characteristics of choroidal melanomas in rabbits.[42] Monitoring the vascular status of the *pecten oculi* is also important for primary or other secondary retinal diseases. For example, atrophy of the *pecten oculi* and its microvasculature were observed in spontaneously occurring inherited retinopathies in chicken, such as *rdd, rge*, and *beg.* [43-45] Additionally, in an experimental setting, degenerative changes of the *pecten oculi* were detected after ablation of its arterial supply.[46]

The objective of present study was to verify if an intravenous contrast solution containing SF6 microbubbles would enhance visualization of the *pecten oculi* during ultrasound examination. Enhanced ultrasonographic visualization of the *pecten oculi* would be beneficial for evaluation of normal structure and function, as well as for diagnosing and monitoring progression of ocular disease. With the variability of the *pecten oculi´*s shapes and sizes across avian species, accurate visualization and precise measurements are critical in making accurate observations and clinical decisions.

2 METHODS

2.1 Animals

All aspects of this study were approved by the Federal University of Paraná's Animal Welfare Committee and were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All birds were apprehended by the Environmental Agency of the State of Paraná (*Instituto Ambiental do Paraná*) located in Curitiba-PR, Brazil, from illegal wildlife trade. Ten adult individuals of the following avian species were selected and investigated: 1 roadside hawk (*Rupornis magnirostris*), 1 stygian owl (*Asio stygius*), 2 striped owls (*Asio clamator*), 2 burrowing owls (*Athene cunicularia*), 2 ring-neck parakeets (*Psittacula krameri*) and 2 domestic chickens (*Gallus gallus domesticus*) of the Cobb 500 strain. Since the wild birds came from a rescue center, the exact age was undetermined; however, the size, weight and anatomical features of all each individual were consistent with sexual maturity. The two chickens (*Gallus gallus domesticu*s) belonged to the Canguiri Experimental farm from the Federal University of Paraná (UFPR), Pinhais-PR, Brazil. The male was 16 months and the female 9 months of age. Specific signalment and weight for each bird included in this study is provided in Table 1. Prior to inclusion in the study, ophthalmic examinations were performed on each subject using a slit lamp biomicroscope (SL-17, KOWA, Tokyo, Japan) and an indirect ophthalmoscope (Heine Omega 180 Headworn Binocular Indirect Ophthalmoscope, Dover, NH, USA) to confirm the absence of ocular abnormalities.

Table 1- Common name, scientific name, sex, estimated age and number of animals included in this study.

Common Name	Scientific Name	Sex	Age	Weight (g)
Roadside Hawk	*Rupornis magnirostris*	Female	Adult	280
Stygian Owl	*Asio stygius*	Male	Adult	660
Striped Owl	*Asio clamator*	Male	Adult	410
Striped Owl	*Asio clamator*	Female	Adult	540
Burrowing Owl	*Athene cunicularia*	Male	Adult	210
Burrowing Owl	*Athene cunicularia*	Female	Adult	240
Ring-Neck Parakeet	*Psittacula krameri*	Male	Adult	120
Ring-Neck Parakeet	*Psittacula krameri*	Male	Adult	130
Chicken	*Gallus gallus domesticus*	Male	Adult	6000
Chicken	*Gallus gallus domesticus*	Female	Adult	5000

2.2 Contrast-Enhanced Ultrasonography

Each animal was sedated with a single intramuscular injection of butorphanol (2 mg/kg) (Torbugesic, Fort Dodge, Campinas-SP, Brazil) and midazolam (1 mg/kg) (Dormire; Cristalia, São Paulo-SP, Brazil). Ten minutes after sedation was done, a 24g catheter was placed in the cutaneous ulnar vein. One drop of proxymetacaine 0.5% (Anestalcon; Alcon® São Paulo, Brazil) was instilled in each eye prior to the ultrasound exam. Ultrasound gel (Carbogel, São Paulo, Brazil) was applied over the corneal surface and a high frequency 16 Mhz B-mode ultrasound transducer (Logiq F6 Ultrasound System, General Electric Company, Madison, WI, USA) was positioned over the superior eyelids of both eyes of each bird, and baseline sonograms in both the transverse and sagittal planes were obtained prior to contrast injection. The *pecten oculi* base was best localized with the transducer dorsally angled with approximately 20-45° of inclination in relation to the corneal surface. After the baseline ocular sonograms were obtained, 4.5 μg/kg of a contrast solution containing SF6 microbubbles (SonoVue, Bracco Imaging, Milano, Italy) was administered intravenously and the ultrasonography of the right eye was immediately performed. Ultrasonography was continued during injection in order to provide real time imaging of the *pecten oculi* throughout intravenous contrast administration and perfusion of the *pecten oculi* vasculature.

2.3 Quantitative ultrasonography image texture parameter analysis

Quantitative image texture parameter analysis of the sonograms was performed using the histogram (statistical class) technique by means of a computer software (MaZda 4.6, Institute of Electronics, Poland), according to the technique described by Castellano et al. (2004).[47] The regions of interest (ROIs) were selected in the exact same area on each ultrasonographic image of the *pecten oculi* before and after the contrast agent was injected. The analysis is based on the limited grey-level values that a pixel may assume, consisting of integer numbers ranging from 0 to 2bK1, where b stands for the number of bits of the image (i.e. this will determine the amount of disk memory occupied by each image pixel). For standard B-mode ultrasonography, 8 bits are used, and therefore the grey-level values range from 0 to 255 (increasing values from dark to lighter pixels). Thus, the resulting units are a count of how many pixels in the image a given grey-level value possess per 8 bits. The analysis was configured using normalization option ±3σ. Image mean value- μ and standard deviation- σ, is computed, and then analysis is performed for grey scale range between μ -3σ and μ +3σ. Texture data of the same ROIs (before contrast injection and after contrast injection) were calculated by the histogram-based method.

2.4 Statistical analyses

Normality testing for ultrasonography texture parameter analysis was performed using the Shapiro–Wilks test,[48] considering alpha = 0.05. The resulting *P* value was 0.867, and the null hypothesis (data distribution was normal) was not rejected. Data were subsequently analyzed using a paired *t*-test for the comparison of pixel intensity values of the same *pecten oculis*´s ROI, before and after contrast injection, in the same animals. All statistical tests were performed using (SigmaPlot 12.5, Systat Software, Inc., San Jose, CA, USA)

3 RESULTS

Visualization of the *pecten oculi* was significantly enhanced by the IV administration of the SF6 microbubble contrast in all subjects. Pre-contrast B-mode ultrasonography revealed the *pecten oculi* projecting anteriorly from the optic disc into the vitreous body. However, delineation of the *pecten oculi* margins were indistinct. Approximately two to three seconds after administration of intravenous contrast, the movement of the microbubbles could be clearly seen inside the *pecten oculi* in the anterior-to-posterior and posterior-to-anterior direction, following a "zigzag" pattern. Microbubble movements were obvious during the initial 45 to 60 seconds, then slowly decreasing until they were no longer apparent at about 2 minutes after injection. The microbubble contrast also enhanced the echogeneticy and thus visualization of the *pecten oculi*. Delimitation of all the *pecten oculi* borders were increased with the gain in contrast against the hypoechoic vitreous body. Contrast enhancement also lasted for approximately 2 minutes. Examples of pre- and post-contrast B-mode sonograms are shown in Figure 1. All subjects recovered normally following sedation without evidence of adverse effects from the use of intravenous contrast.

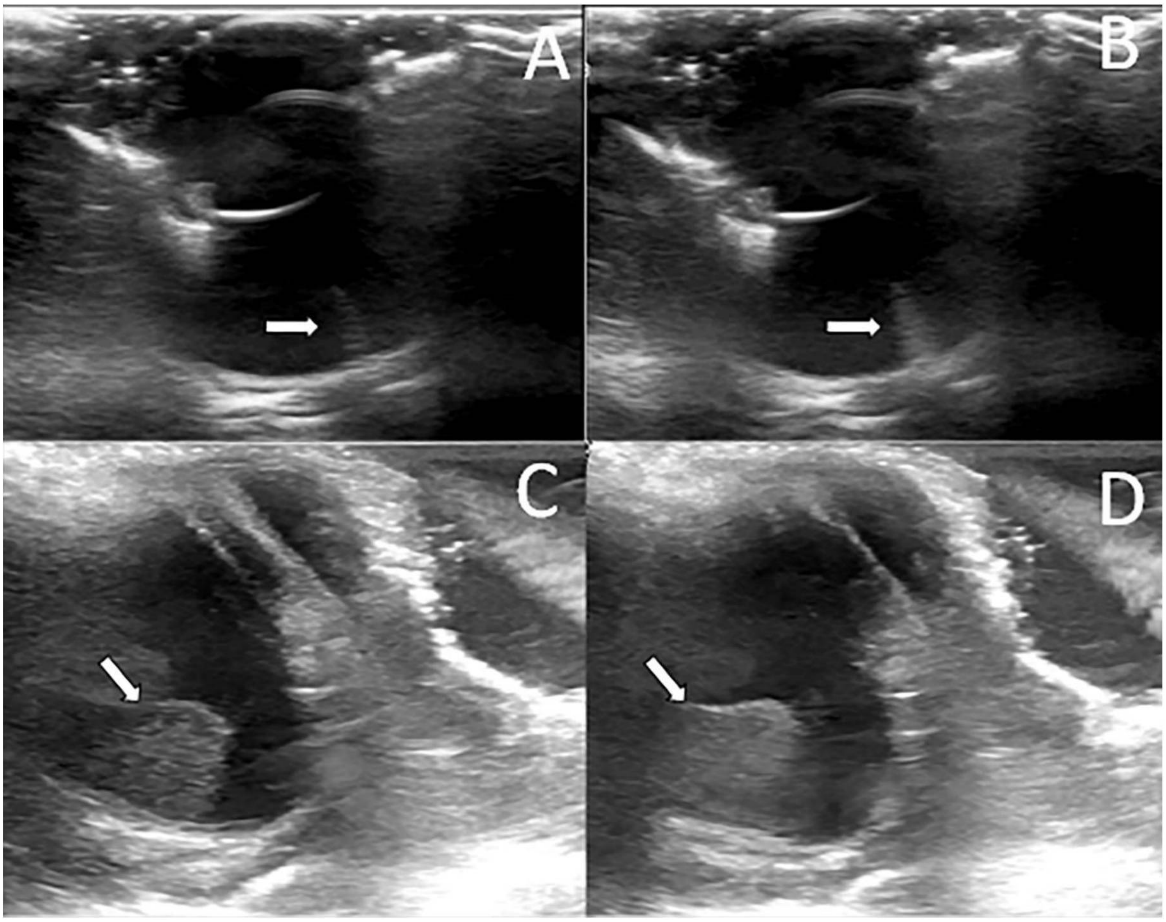

Figure 1. Baseline and contrast-enhanced ultrasonography of A,B) the sagittal plane of the right globe of a roadside hawk (*Rupornis magnirostris*), and C,D) the transverse plan ultrasonography of the left globe of a burrowing owl (*Athene cunicularia*). A and C represent baseline sonograms prior to SF6-based contrast injection, and B and D represent sonograms obtained 3 seconds after intravenous injection of contrast and shows increased echogenicity of the *pecten oculi* due to contrast enhancement. The cornea is located towards the top and to the right in A and B, and towards the top and to the left in C and D. The white arrows indicate the *pecten oculi*.

In the quantitative image texture analysis, all histograms from the *pecten oculi*´s ROIs presented a significant (*P*=0.002) increase in pixel intensity values. In other words, it was possible observe quantitatively that *pecten oculi* images gained in number of pixels, thus, attaining a lighter tonality after receiving the microbubbles contrast injection (see Figure 2, Table 2).

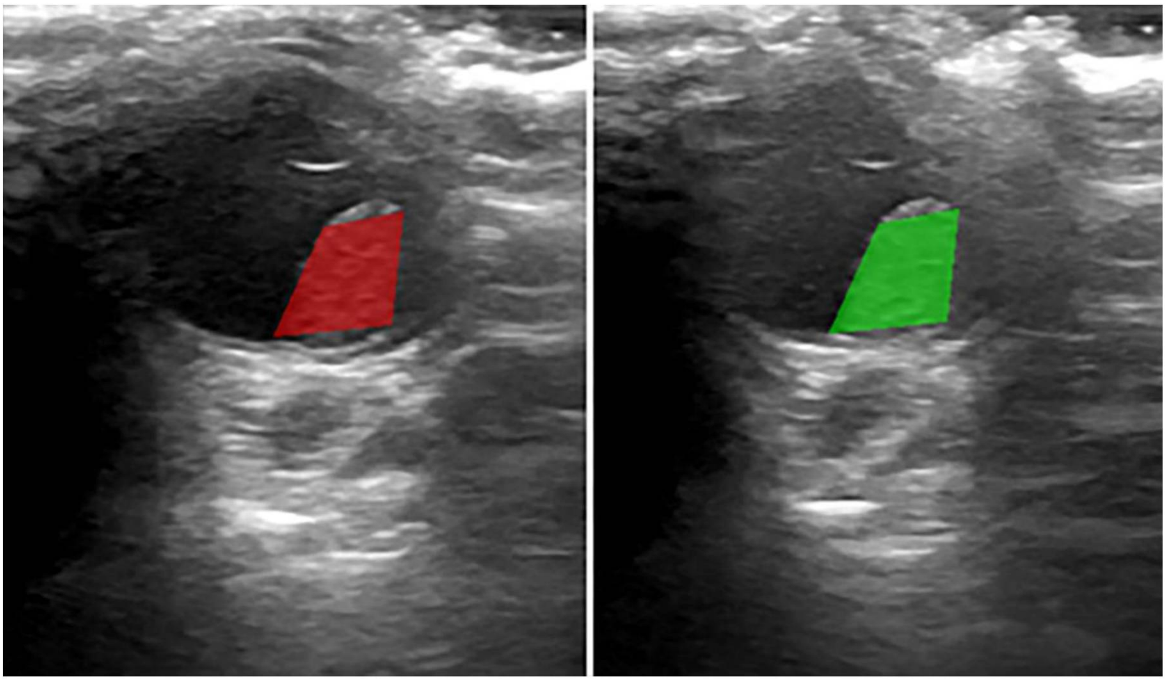

Figure 2. Representative sonograms of the right *pecten oculi* of a ring-neck parakeet (*Psittacula krameri*) showing image texture comparison being performed with the computer software (MaZda 4.6, Institute of Electronics, Poland) by means of the histogram (statistical class) technique. Measured portions of the *pecten oculi* were selected in the exact same anatomical region on each ultrasonographic image, before (red area) and after (green area) the contrast agent was injected.

Table 2. Quantitative image texture parameter analysis based on pixel values obtained of the *pecten oculi* in all avian species examined, before and after intravenous injection of SF6-based contrast.

***Pecten oculi*´s pixel intensity**	**Before IV contrast**	**After IV contrast**
Mean	53.89	75.78*
Median	57.15	81.45
Standard Deviation	16.12	21.78
Minimum	29.27	42.62
Maximum	72.87	115.94
Variance	259.70	474.30
Skewness	-0.36	-0.02
Kurtosis:	1.516	2.45
Interquartile Range (IQR)	22.18	17.76

*Significantly diffrent, *P*=0.002

4 DISCUSSION

This work describes for the first time live ultrasonographic imaging of the *pecten oculi* in birds using microbubble CE-US. Increased visualization of the pecten oculi was achieved in all subjects following administration of an SF6 microbubble contrast solution, with sharper delineation of the boundaries of the *pecten oculi*. Intravenously administered contrast material took only a few seconds (2-3) to increase visualization of the pecten oculi ultrasonographically, and increased visualization was evident for about 2 minutes. Even though the anterior-to-posterior and posterior-to-anterior movements of the microbubbles were easily visualized during ultrasonography and in the recorded videos, it is a feature unfortunately not quite captured in the still images of the sonograms presented in this study. However, visualization of the microbubble flow made possible to delimitate the position of the innumerous ascending and descending capillaries that constitute the vascular framework of the *pecten oculi*.[10] In the future this contrast agent might be used to check patency and integrity of these blood vessels in ocular disease. Additionally, the *pecten oculi* microvasculature was not permeable to SF6 contrast agent, as no contrast material or microbubbles were detected in the vitreous body, an observation that supports the participation of the *pecten oculi* as a part of the blood-ocular barrier.[19,20,48,49] This finding is particularly relevant as other contrast-enhancing imaging modalities (e.g. fluorescein angiography) on the eyes of birds have shown leakage of contrast from the *pecten oculi* into vitreous following intravenous administration.[20,21]

Previous studies showing success of SF6 microbubble enhancement of the microvasculature in the eyes of small animals such as rabbits and mice, combined with the results shown here of successful enhancement of the *pecten oculi* in several avian species, is encouraging when considering the small eyes and delicate microvasculature of many avian species.[37,42] Enhanced visualization of the *pecten oculi* and its vasculature using a readily available diagnostic imaging method such as CE-US would facilitate future studies evaluating the role of the *pecten oculi* in posterior segment diseases, including retinal degenerations, such as *rdd*, *rge* and *beg*.[43-46] In the future, CE-US could be employed in the diagnosis of *pecten oculi* abnormalities as well as in the monitoring of progression vs. healing in pathological processes. This is particularly relevant in eyes where funduscopic visualization of the pecten is not possible (e.g. cornea disease, cataracts, inability to achieve sufficient mydriasis, etc.). Overall, the use of SF6 microbubble CE-US in birds is safe, easy to perform, and provides increased visualization of a delicate intraocular structure that is of particular research interest is of clinical importance in avian ocular disease.

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CAPÍTULO II

Antimicrobial activity of topical dyes used in clinical veterinary ophthalmology

Antimicrobial activity of topical dyes used in clinical veterinary ophthalmology

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Abstract

Objective: To evaluate *in vitro* the antibacterial effects of fluorescein, rose bengal, and lissamine green topical ophthalmic dyes against selected Gram-positive and Gram-negative bacteria, and to evaluate if preservative or preservative-free fluorescein solutions are able to inhibit or potentiate bacterial growth.
Procedures: Susceptibility testing was performed using the Kirby-Bauer disk diffusion method plated with clinical ocular isolates of *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Streptococcus* spp., *Escherichia coli*, and *Pseudomonas aeruginosa.* Bacterial growth inhibition was evaluated 24 hours following the addition of commercially available fluorescein, rose Bengal, and lissamine green sterile strips. Antimicrobial effectiveness testing was performed by inoculation of compounded 1% dye solutions, both with and without preservatives (fluorescein and lissamine contained thiomersal, and rose bengal contained nipagin and nepazol), with the five previously mentioned bacteria. Growth was evaluated at days 7, 14, and 28.

Results: All dyes showed antibacterial activity against Gram-positive organisms. Preservative-free compounded 1% fluorescein solution inhibited growth of Gram-positive organisms but not of Gram-negative organisms. Preservative-free rose bengal and lissamine green inhibited growth of both types of organisms.

Conclusions: Ocular surface samples for antimicrobial culture should be preferably taken prior to the administration of topical dyes, due to potential antibacterial activity. This suggestion is stronger if undiluted strips are directly applied or when commercial fluorescein solutions are used and not immediately rinsed. Ophthalmic dye solutions containing preservative are safe from bacteria growth for up to 28 days if properly handled and stored. The use of preservative-free fluorescein solutions should be avoided and preservative-free rose bengal and lissamine green should be handled carefully.

Key words: Fluorescein, rose bengal, lissamine green, antimicrobial activity, eye drops, *Pseudomonas aeruginosa*

1 INTRODUCTION

Fluorescein, rose bengal, and lissamine green are the main topical biological dyes used for diagnosis of ocular surface disease in the practice of medical and veterinary ophthalmology.[1-3] Fluorescein is primarily used for diagnosing corneal ulcers and conjunctival epithelial defects, and for evaluating the tear film breakup time. It is also used to assess the presence of aqueous humor leakage (Seidel test) and to investigate the patency of the nasolacrimal system (Jones test).[1] Rose bengal is used to evaluate the corneal tear film and surface epithelial defects where both healthy and non-healthy cells are stained if there is a breach in the tear film. Lissamine green, although similar in staining pattern, does not stain healthy cells regardless of tear-film quality.[1-4]

All three dyes are available in impregnated sterile paper strips, which are generally considered the safest and preferred formulation.[5] The impregnated strips can be quickly prepared as a solution by mixing the impregnated paper strip with a sterile saline solution inside a syringe.[4] Fluorescein is also available as a solution in a dropper form. Multiple-use topical solutions are at risk of contamination when touched to the ocular surface, cilia, facial hairs or any other structure during administration. Unintentional contamination could result in transmission of microorganisms from one patient to the next since the same bottle of solution is used on multiple animals, which could potentially give rise to ocular surface infections.[6-8]

Cross-contamination of commercial topical fluorescein solution and consequent ocular infection caused by *Pseudomonas aeruginosa* has been reported.[6,9] Although not an ideal culture medium (i.e. containing proper nutrients – oxygen, carbon, nitrogen, inorganic phosphate and sulfur, trace metals, etc. as well as appropriate pH), minimal medium requirements can be met within a topical fluorescein solution following contamination (i.e. carbon source, salts, water).[10] Indeed, bacteria (i.e. *Pseudomonas aeruginosa*) has been shown to survive, but not growth, in fluorescein 2% solution by a year or more despite the presence of antimicrobial preservatives.[11] No studies have shown that fluorescein solutions directly promote bacterial growth, but interestingly, certain strains of *P. aeruginosa* are able to produce fluorescent particles of their own, or siderophores called pyoverdines, which are important virulence factors.[12,13] Claoué (1986) studied the use of preservative-free eye drops containing 2% of fluorescein contaminated by *Pseudomonas aeruginosa,* and although it was suggested that use of such as solution was relatively safe, this study only evaluated the solution for 24 hours post-contamination without quantitative analyses.[14] It is not known whether bacterial microorganisms could contaminate rose bengal and lissamine green solutions given that they do not come available in a commercial solution.

On the contrary, rose bengal has been shown to have bacteriostatic effects in fungal culture media, and lissamine green exhibits antiviral activity.[15-17] Additionally, fluorescein has been shown to have antibacterial activity against *Moraxella catarrhalis, Streptococcus pneumoniae*, and *Haemophilus influenza* (although not against *Pseudomonas aeruginosa* and *Staphylococcus aureus*).[18] Both rose bengal and fluorescein had bacterial growth inhibition, although discrete, for *Staphylococcus aureus.*[19] This is an important consideration when planning the order of diagnostic testing as administration of a topical dye prior to obtaining culture or cytology could in theory impact the results.

The purpose of the present study was two-fold. Our first aim was to investigate the impact on growth of three Gram-positive and two Gram-negative bacteria species by three commonly used ocular surface dyes (fluorescein, rose bengal, and lissamine green), using the Kirby-Bauer disk diffusion antibiotic sensitivity test.[20-22] Our second aim was to evaluate whether or not the same three ocular surface dyes could inhibit or potentiate growth of the same bacteria in solutions with or without preservative by means of antimicrobial effectiveness testing.

2 MATERIAL AND METHODS

2.1 Sensitivity testing (modified Kirby–Bauer test)

Antibiotic susceptibility plates containing a non-selective, non-differential microbiological growth medium (Mueller–Hinton medium) and a differential media to fastidious organisms (Blood agar) were used, each having a depth of media of 4 mm. Plates were gently streaked in a side to side motion with sterile swabs (according to the Kirby–Bauer method[20]) containing a pure culture inoculum of the following clinical isolates: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus pseudintermedius,* and *Streptococcus* spp*.* All strains were isolated from dogs with ocular surface disease. *Staphylococcus aureus* was isolated from a conjunctivitis case caused by a foreign body. *Escherichia coli* was isolated from a conjunctivitis case caused by dacryocystitis. *Pseudomonas aeruginosa* was isolated from a malacic corneal ulcer. *Staphylococcus pseudintermedius* was isolated from a case of keratoconjunctivitis sicca. *Streptococcus* spp. was isolated from a superficial corneal ulcer. Bacteria were identified based on colony morphology and biochemical tests. *Staphylococcus pseudintermedius* was identified by *matrix-assisted laser desorption/ionization-time of light* (*MALDI-TOF*) (Thermo Fisher Scientific, Waltham, USA). The inoculum was standardized by the McFarland method, prepared in a 20 ml screw cap tube with 0.1 ml of 1% barium chloride and 9.9 ml of sulfuric acid, giving a McFarland 1.0 standard. Each colony was touched by a loop and transferred to sterile tubes containing brain heart infusion broth. The tubes were incubated at 35-37°C until the turbidity was the same or similar to the MacFarland 1.0 standard or 3×10^8 CFU/ml. The pH level of the agar was set between 7.2 and 7.4.

Fluorescein, rose bengal, and lissamine green sterile strips (Drogavet, Curitiba, Brazil) were applied in sterile fashion to each of the plates as would be an antibiotic strip using the Kirby-Bauer method. The strips contained 3 different amounts (0.01 mg, 0.1 mg, 1.0 mg) of fluorescein sodium USP, rose bengal, and lissamine green. Strips containing 0.01 mg and 0.1 mg were specially compounded, and strips containing 1.0 mg are commercially available. Sterile strips with no dyes were applied to serve as negative controls. Plates were incubated at 30-35ºC for 24 hours and were then evaluated for the presence of a clear zone around strips indicating growth inhibition of the cultured bacteria by the corresponding dye.[20-22] If present, the width of an inhibitory zone around the sterile strip was measured (with a ruler). These tests were repeated in triplicates. Results were reported and interpreted similarly as if the dyes in the ophthalmic sterile strips were antibiotics in sterile discs. In order to establish parameters for interpreting antibacterial activity, our results were compared to a pool of published Kirby-Bauer test results (mean inhibitory zone width) used to analyze typical antibiotics tested against the same microorganisms. [23-26] A total width of 5 mm is considered to have no bacterial growth inhibition because this is the width of the dye-impregnated sterile strips tips. If the width of inhibition zones ≤10 mm = resistant; width 10–19 mm = intermediate susceptibility; width ≥20 mm = susceptible to the antimicrobial activity. As in a regular Kirby-Bauer test, the measurement of the zone of inhibition included the width of the paper strip. Zones of inhibition measurements were based on CLSI VET08 and varied according to the bacterial species and antimicrobial action or effectiveness. A possible antimicrobial mechanism of biological dyes, as well as its dosage and concentration have not been well-described, so the magnitude of inhibition zones (in millimeters) were based on minimum values to determine resistance and maximum values to determine sensitivity.

2.2 Antimicrobial effectiveness testing

In addition to testing 3 different dilutions of each dye, we also tested solutions with (A) and without (B) preservative. These tests were performed in duplicates. Preservative-free 1% solutions were compounded specially. Commercially available and compounded 1% solutions of each of the dyes containing preservatives were used: 1) Fluorescein - 400 µg/ml of thiomersal (Allergan, São Paulo, Brazil); 2) Rose bengal – 0.3 mg/ml of nipagin and 0.6 mg/mg of nepazol (compounded by Drogavet, Curitiba, Brazil).; 3) Lissamine green - 400 µg/ml of thiomersal (compounded by Drogavet, Curitiba, Brazil). A total of 6.93 ml of each solution was used. Both Solutions A and B were confirmed to have a starting bacterial concentration of less than 10 CFU/ml, considering this value as the limit of bacterial detection. Solution A was administered into five sterile bacteriological containers (7 ml each). Standardization of the inoculum was followed as previously described above. One of each container was inoculated with 0.07 ml of 3 x 10^8 CFU/ml (McFarland 1.0 standard) of either a Gram-positive bacterium, *Staphylococcus aureus*, *Staphylococcus pseudintermedius* or *Streptococcus* spp., or a Gram-negative organism, *Escherichia coli* or *Pseudomonas aeruginosa*, respectively. Counting was performed on two plates to determine the resulting inoculum of 3 x 10^6 CFU/ml mixed with the solutions. The containers were stored at 22.5 ± 2.5°C and CFU/ml was calculated at days 7, 14, and 28. Solution B received the same inoculum at the same concentrations under the same environmental conditions. CFU/ml was calculated at days 7, 14, and 28. The number of bacteria (CFU/ml) at each time point was calculated by multiplying the number of colonies by the dilution factor. To determine the posterior CFU/ml count, 0.07 ml of inoculate was spread over two plates, and the number of CFUs were counted and averaged. The same containers were sampled at all timepoints.

2.3 Statistical Analysis

Both descriptive and inferential statistics were used. Descriptive statistics was used to demonstrate the results for antimicrobial effectiveness testing. Zone of inhibition width in the sensitivity test was normally distributed according to the Shapiro-Wilk test, had a symmetry of 0.19 (i.e. less than 1.5), and kurtosis less than 2. Subsequently, a 3 × 5 × 3 factorial analysis was applied to capture the effect of the dyes (3 levels), bacteria (5 levels), and concentrations (3 levels) as well as their respective interactions. A Tukey's HSD All-Pairwise Comparisons Test was subsequently applied for the means with significant differences. *P*-values < 0.05 were considered significant. Statistix 7 9.0 (Statistix 9.0 Analytical Software, Tallahassee, Florida, USA) software was used for all analyses.

3 RESULTS

3.1 Sensitivity testing (modified Kirby–Bauer test)

Antibiotic sensitivity testing revealed that commercially available sterile fluorescein, rose bengal, and lissamine green strips presented different zones of inhibition depending on the concentration and bacterial species (Figure 1). Considering the zone of inhibition width, the interaction between type of bacteria and type of dye (*P* = 0.0001), and the dye amount (*P* = 0.0002), were both significant. However, the interaction between dye amount and type of dye was not significant (*P* = 0.37). Lastly, the interaction between the type of bacteria, the type of dye, and the dye amount also was not significant (*P* = 0.95). Zones of inhibition and descriptive and inferential statistics are presented in the Table 1.

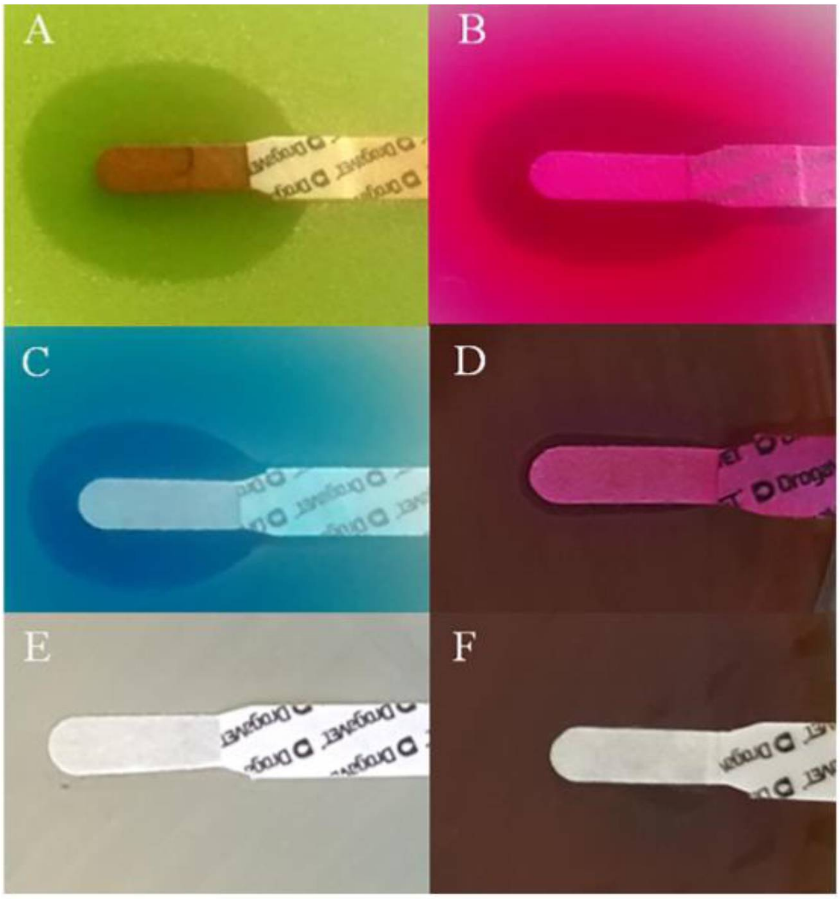

Figure 1. Antibiotic sensitivity test using sterile strips with different ophthalmic dyes. Large and clear inhibition zones (haloes) near the tip of the sterile strips denotes bacterial growth inhibition. The measurement of the zone of inhibition included the width of the paper strip. (A) Sterile paper strip with 1 mg fluorescein producing a zone of inhibition in a S. pseudintermedius culture in Mueller–Hinton medium. (B) Sterile paper strip with 0.1 mg rose bengal producing a zone of inhibition in a S. aureus culture in Mueller– Hinton medium; (C) Sterile paper strip with 0.1 mg lissamine green to S. aureus producing a zone of inhibition in Mueller–Hinton medium; (D) Sterile paper strip with 0.01 mg rose bengal producing a very discrete zone of inhibition in a Streptococcus spp. culture in blood agar medium; (E) Negative control to S. aureus in Mueller–Hinton medium; (F) Negative control to Streptococcus spp. in blood agar medium.

Table 1. Mean zone of inhibition in the antibiotic sensitivity tests of different concentrations of fluorescein, rose bengal, and lissamine green sterile ophthalmic strips for five different bacterial species.

Bacteria	**Control**		**Fluorescein**		**Rose Bengal**		**Lissamine green**		**Fluorescein**		**Rose Bengal**		**Lissamine green**		**Fluorescein**		**Rose Bengal**		**Lissamine green**	
	1 mg (concentration)								**0.1 mg (concentration)**						**0.01 mg (concentration)**					
	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
S.aureus	0	0	19.667^{A}	0.577	23.333^{B}	0.577	20.667^{AC}	0.577	13.667^{A}	0.577	20.667^{B}	0.577	19.667^{BC}	0.577	7.333^{A}	0.577	8.333^{AB}	0.577	8.333^{BC}	0.577
P.aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E.coli	0	0	0^{A}	0	10.333^{B}	0.577	9.333^{BC}	0.577	0^{A}	0	9.667^{B}	0.577	9.333^{BC}	0.577	0	0	0	0	0	0
S.pseudintermedius	0	0	8.333^{A}	0.577	22.333^{B}	0.577	20.667^{C}	0.577	4.333^{A}	0.577	18.667^{B}	0.577	19.667^{BC}	0.577	0^{A}	0	7.667^{B}	0.577	6.667^{BC}	0.577
Streptococcus spp.	0	0	0^{A}	0	7.333^{B}	0.577	6.667^{BC}	0.577	0	0	7.333	0.577	6.667	0.577	0^{A}	0	1.333^{B}	0.577	1.333^{BC}	0.577

Legend: Std. Dev. - standard deviation

Mean values in the same line of dye concentration, under the same concentration, with different uppercase letters indicate significant differences for each bacterium, respectively.

3.2 Antimicrobial effectiveness testing

Antimicrobial effectiveness testing of solutions with preservative showed a decrease in the concentration of all bacterial inoculum (from 3.0 x 10^8 to <100 CFU/ml) after 7 days. Solutions without preservative had varying results. Primarily, fluorescein solution had a decrease in the inoculum concentration of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in the first seven days, whereas *Pseudomonas aeruginosa* and *Escherichia coli* showed a slight increase in concentration. From 14 to 21 days, *Staphylococcus aureus* and *Staphylococcus pseudintermedius* showed continued reduction in concentration to <100 CFU/ml while reduction of *Streptococcus* spp. was less intense but decreased to <100 CFU/ml by the 14th day. *Pseudomonas aeruginosa* and *Escherichia coli* grew exponentially. Lissamine green and rose bengal decreased slightly the concentration of *Pseudomonas aeruginosa* and *Escherichia coli* in the first seven days, but more intense reduction was seen after 14th day until <100 CFU/ml. Other bacterial species reduced to <100 CFU/ml in the first seven days. All values are presented in Table 2. None detected was indicated by “ND” in table 2 when the value presented to <100 CFU/ml.

Table 2. Antimicrobial effectiveness testing of all dyes with or preservative-free. Acceptable initial count < 10 CFU/ml. Initial inoculum was standardized in 3.0×10^{8} CFU/ml.

	Bacteria (cfu/g)	**Count after 7 days**	**Count after 14 days**	**Count after 28 days**
Fluorescein 1%	*S.aureus*	ND	ND	ND
	P.aeruginosa	ND	ND	ND
	E.coli	ND	ND	ND
	S.pseudintermedius	ND	ND	ND
	Streptococcus spp	ND	ND	ND
Fluorescein 1% PF	*S.aureus*	1.8×10^{4}	ND	ND
	P.aeruginosa	4.2×10^{8}	2.0×10^{9}	2.2×10^{10}
	E.coli	4.1×10^{8}	1.6×10^{9}	2.8×10^{10}
	S.pseudintermedius	5.4×10^{4}	ND	ND
	Streptococcus spp	6.8×10^{7}	7.2×10^{4}	ND
Rose Bengal 1%	*S.aureus*	ND	ND	ND
	P.aeruginosa	ND	ND	ND
	E.coli	ND	ND	ND
	S.pseudintermedius	ND	ND	ND
	Streptococcus spp	ND	ND	ND
Rose Bengal 1% PF	*S.aureus*	ND	ND	ND
	P.aeruginosa	5.1×10^{7}	ND	ND
	E.coli	3.4×10^{6}	ND	ND
	S.pseudintermedius	ND	ND	ND
	Streptococcus spp	ND	ND	ND
Lissamine G 1%	*S.aureus*	ND	ND	ND
	P.aeruginosa	ND	ND	ND
	E.coli	ND	ND	ND
	S.pseudintermedius	ND	ND	ND
	Streptococcus spp	ND	ND	ND
Lissamine G 1% PF	*S.aureus*	ND	ND	ND
	P.aeruginosa	$6,4 \times 10^{6}$	ND	ND
	E.coli	$5,5 \times 10^{6}$	ND	ND
	S.pseudintermedius	ND	ND	ND
	Streptococcus spp	ND	ND	ND

Legend: PF-preservative-free. ND – None Detected

All dyes presented initial count < 10 CFU/ml.

Initial inoculum was standardized in 3.0×10^{8} CFU/ml.

4 DISCUSSION

Fluorescein (resorcinolphthalein) and rose bengal (di-sodium-tetra-iodo-tetra-chlorfluorescein) are classified as xanthine dyes, whereas lissamine green is a diphenyl-naphthyl methane derivative of the phenylmethane dye. Fluorescein, rose bengal, and lissamine green have been shown to penetrate living cells affecting cellular metabolism in different degrees.[16,17] There are commercially available dye strips containing different amounts of fluorescein, rose bengal, and lissamine green. Examples of commercial biological dyes and their amounts include: 1) fluorescein strips 1.0 mg (I-DEW FLO, ERC,UK), (OptiStrips-FL, Optimed, AU) or 0.6 mg (GloStrips, AMCON, MO, USA); 2) rose bengal strips 1.3 mg (BioRose, Biotech, Switzerland), (GloStrips, Amcon, USA); 3) Lissamine green strips 1.5 mg (I-DEW Green, ERC, UK), (GreenGlo, AMCON, MO, USA). Fluorescein is the only topical dye with a commercially available eye drop solution (Sodic Fluorescein 1%, Allergan, São Paulo, Brazil).

Overall, the results provided in the present study show that commercially available sterile ophthalmic strips containing fluorescein, rose bengal, and lissamine green stains have variable antibacterial activity against three Gram-positive organisms (*Staphylococcus aureus*, *Staphylococcus pseudintermedius,* and *Streptococcus* spp.) but only weak or no activity against two Gram negative organisms (*Escherichia coli* and *Pseudomonas aeruginosa*). Additionally, antimicrobial effectiveness testing of solutions containing preservative showed a decrease in all bacterial inoculum, whereas variable antimicrobial effects were noted for solutions without preservative, importantly the continued growth of Gram-negative organisms (*Escherichia coli* and *Pseudomonas aeruginosa*). Testing the concentration of the preservatives alone would have helped to differentiate the effects of the topical dye from the preservative alone. This certainly is a limitation of the study. However, since preservative-free and preservative containing dye were tested, we believe the readers would be able to infer from this.

A previous investigation showed that sterile ophthalmic strips containing 1 mg of fluorescein possess antibacterial activity against the Gram-positive bacteria *Streptococcus pneumonia*.[18] A similar study evaluated the effect of topical ophthalmic solutions containing 1% fluorescein, 1% rose bengal, and 1% lissamine green on bacterial colony growth of *Staphylococcus aureus* (ATCC 25924), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 1388) and

Streptococcus pneumoniae (ATCC 49619).[19] This work corroborates our findings showing that growth of *E.coli* and *P. aeruginosa* is not effectively inhibited by any dye tested, but also that rose bengal and fluorescein are able to inhibit the growth of *Staphylococcus aureus.* Lissamine green did not inhibit bacterial growth for any of the species tested.[19] In our study, however, lissamine green was able to generate zones of inhibition to *Staphylococcus aureus*, *Staphylococcus pseudintermedius* and *Streptococcus* spp. suggesting that lissamine green in fact does have antibacterial activity to at least two bacteria frequently found on the ocular surface. The differences between our and previous results may be related to a difference in formulation and/or concentration of lissamine green used in these two investigations (1% ophthalmic solution versus a paper strip containing 1.0 mg) or small differences in laboratory conditions (agar thickness, pH).[19]

Different patterns of growth were noted between antibiotic sensitivity testing and antimicrobial effectiveness testing among the different bacteria. For example, zones of inhibition were not noted with any dye for *Pseudomonas aeruginosa*, however bacterial concentration decreased even in preservative free rose bengal and lissamine green solutions. One possible explanation is that the concentration of dye solution is contributing to the inhibition of bacterial growth, which was supported in this study. The concentration of the dye solutions was ten times greater than that of the strips, which could explain the decline in growth in preservative free rose bengal solutions. However, *Pseudomonas aeruginosa* and *Escherichia coli* CFUs both increased in preservative free fluorescein sodium solution up to 28 days. A second possible cause could be the lack of nutrients in dye solutions over time to maintain a bacterial population and sustain their growth, compared to culture plates that provide suitable nutrients. Specific nutritional requirements are found in different bacterial species like *Streptococcus* spp., of which grow only on blood agar plates or in THY liquid medium, and thus the lack of nutrients available could result in decreased growth over time.[28] The opposite occurred with *Escherichia coli and Pseudomonas aeruginosa* in fluorescein solution, where there was increase of bacterial CFUs. This could be due to the presence of the enzyme resorcinol 4-hydroxylase, which could metabolize fluorescein (resorcinolphthalein) to be used as a source of carbon, hydrogen, and oxygen.[29]

The mechanism of how ophthalmic dyes kill some species of bacteria but not others is still uncertain. It has been suggested that rose bengal and fluorescein could cause photosensitization by a series of complex physical and biochemical cascades ultimately resulting in antibacterial and antiviral activities. [30-33] Even though the precise mechanism for bacterial toxicity of lissamine green is not fully understood, it is known that this dye has the ability to inhibit herpes simplex virus type 1 plaque formation in Vero cells, *in vitro*, correlating with suppression of cellular metabolism.[16] Nevertheless, unlike rose bengal, lissamine green seems to not interfere with viral replication *in vivo*.[34] Additionally, lissamine green has an excellent safety profile and has not been shown to stain healthy ocular surface cells and has a minimal effect on cell viability.[16,34]

There currently is no standardization of the zone of inhibition width parameters for the different sterile dye strips used in this study, with variability based on different bacterial species, antibiotics, culture media, and environment conditions.[27] We selected performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals,[27] and averaged and compared them to the ones found in our investigation for each bacterial species. According to our results, only *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in rose bengal and lissamine green strips containing greater than 0.1 mg were properly inhibited. Fluorescein inhibited *Staphylococcus aureus* in strips containing 1.0 mg. Other findings showed intermediate inhibition, or resistance to topical dyes (most prevalent among the Gram-negative organisms).

One potential point of variability could be in the amount of dye reaching the eyes at the time of application of the strips. Snyder and Paugh (1998) measured the amount of rose bengal that was released in eye models after a given soak time in balanced saline solution. They found variable values depending on the soak time, with the maximum value reaching 10.27×10^{-6} g/ml (0.01 mg/ml) in 45 seconds of immersion and minimum value being 7.64×10^{-6} g/ml (0.007 mg/ml) in 15 seconds of immersion. This suggests that the amount of strip dye reaching the ocular surface could be insufficient to promote bacterial growth inhibition. However, in the same study, the first drop from a wetted strip was rejected, an instruction that is unclear in some clinical papers and missing from most package instructions of commercial strips.[4,35-37] Nevertheless, whether or not the first, hypothetically more concentrated, drop could reach sufficient concentration to inhibit bacterial growth is also uncertain. Although it seems unlikely that sufficiently high concentrations of biological dyes would be achieved at the ocular surface to inhibit bacterial growth during a routine ophthalmic examination, it is recommended that any samples for bacterial cultures be collected prior to dye application, especially if undiluted paper strips are directly applied to the ocular surface or when fluorescein as an eye drop solution is used and not immediately rinsed.

Previous anecdotal reports suggest that fluorescein could support bacterial growth despite the presence of common preservatives such as benzalkonium chloride and chlorobutanol.[9] Therefore, only disposable sources (e.g., impregnated paper strips, single-dose vials) of topical ophthalmic stains were recommended. However, we showed that growth of all bacteria was restricted in commercially and compounded available topical ophthalmic preparations of 1% sodium fluorescein, rose bengal, and lissamine green containing preservative for up to 28 days. These findings suggest that topical ophthalmic dye solutions containing preservative are safe for up to 28 days.

Growth of Gram-positive organisms was not detected in a compounded 1% sodium fluorescein solution despite the absence of preservatives, whereas Gram-negative organisms did exhibit growth. However, growth of *Pseudomonas aeruginosa* and *Escherichia coli* did not exhibit a typical exponential growth phase at 24 to 48 hours, but rather demonstrated slow growth (i.e. only after 14 days).[39,40] Therefore, this solution did not behave as a classic culture medium, but similar to inoculation of bacteria in water.[41] Even though a 1% preservative-free sodium fluorescein solution did not act as a good culture medium, *Escherichia coli* and *Pseudomonas aeruginosa* were still able to grow, and thus additional care should be taken when using such formulation, particularly at the time of administration to a patient. It is not recommended to use a multi-dose dispenser of this formulation beyond 7 days after opening, even when storing it inside a refrigerator. Additionally, in-house fluorescein solutions made inside a syringe should also be used with caution as they do not contain a preservative and could support growth of Gram-negative bacteria. Many ophthalmologists prepare fluorescein solutions using a topical anesthetic rather than a balanced salt solution, which may have the ability to quickly regain sterility after contamination (i.e. minutes to hours).[41] However, only two bacterial species were evaluated and bacterial growth over a longer period of time remains unknown, thus care should be taken when storing fluorescein-anesthetic combinations as with other compounded formulations.[41,42]

Our results support the use of commercial and/or manipulated solutions of 1% sodium fluorescein, rose bengal and lissamine green containing the preservative for at least 28 days, whereas the use of preservative-free compounded 1% sodium fluorescein should be avoided and use of preservative-free rose bengal and lissamine green should be handled carefully.

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ANEXO I

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ORIGINAL ARTICLE

WILEY

The use of sulfur hexafluoride microbubbles for contrast-enhanced ocular ultrasonography of the *pecten oculi* in birds

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Abstract

Background: The *pecten oculi* is a vascular and pigmented structure localized within the posterior segment of all avian eyes. Its primary function is not fully understood yet.

Objective: As ultrasonography (US) is a useful imaging modality for evaluation of the *pecten oculi*, the objective of this study was to investigate the utility of an intravenous contrast solution of sulfur hexafluoride (SF6) microbubbles as a means of enhancing visualization of the *pecten oculi* in normal birds.

Animals studied: Ten adult individuals of the following avian species were evaluated: 1 roadside hawk (*Rupornis magnirostris*), 1 stygian owl (*Asio stygius*), 2 striped owls (*Asio clamator*), 2 burrowing owls (*Athene cunicularia*), 2 ring-necked parakeet (*Psittacula krameri*), and 2 domestic chickens (*Gallus gallus domesticus*).

Procedure(s): After baseline ocular sonograms were obtained in sedated animals, 4.5 μg/kg of a contrast solution containing SF6 microbubbles was administered intravenously and US of the right eye was immediately performed. US was continued during injection to provide real-time imaging of the *pecten oculi* during vascular perfusion of contrast material. *Results*: Within 2-3 seconds following intravenous contrast administration, microbubbles reached the *pecten oculi* of all birds investigated and provided significant ultrasonographic contrast enhancement.

Conclusions: SF6 microbubble contrast ultrasonography in birds is a safe and easy procedure that provides increased contrast and enhanced visualization of the *pecten oculi*. Future use may enable further discovery of its physiologic functions and aid in the development of therapeutic plans for avian intraocular disease.

KEYWORDS
avian, diagnostic imaging, eye, ophthalmology, retina

1 | INTRODUCTION

The avian eye has several distinctive anatomical and physiologic features when compared to the eyes of other vertebrate taxa.[1] A large variability in eye shape, mechanisms of both lenticular and corneal accommodation, variable numbers and positions of retinal specializations, and the presence of the *pecten oculi* are among the most conspicuous features.[1-5] The pecten oculi is of particular interest as there is currently no consensus for its most important physiologic role.[6-8] It is a pigmented and highly vascularized intraocular structure projecting anteriorly from the optic