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Marlane Geribone Seeger

ATIVIDADE DE PORFIRINAS TETRA-CATIÔNICAS FRENTE A ISOLADOS E BIOFILMES DE *Moraxella* spp. ENVOLVIDAS NA CERATOCONJUNTIVITE INFECCIOSA BOVINA

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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Medicina Veterinária Preventiva da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Mestre em Medicina Veterinária.**

Orientador: Prof. Dra. Juliana Felipetto Cargnelutti

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Marlane Geribone Seeger

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Juliana Felipetto Cargnelutti, Dra. (UFSM)
(Presidente/Orientador)

Grazieli Maboni, Dra. (University of Guelph)

Letícia Trevisan Gressler, Dra. (IFFar-FW)

Santa Maria, RS
2022

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RESUMO

ATIVIDADE DE PORFIRINAS TETRA-CATIÔNICAS FRENTE A ISOLADOS E BIOFILMES DE *Moraxella* spp. ENVOLVIDAS NA CERATOCONJUNTIVITE INFECCIOSA BOVINA

AUTORA: MARLANE GERIBONE SEEGER
ORIENTADOR: JULIANA FELIPETTO CARGNELUTTI

A ceratoconjuntivite infecciosa (CIB) é a doença ocular mais importante na criação de ruminantes em todo o mundo e, embora não seja fatal, pode causar grandes perdas na produção. O agente primário da doença é a *Moraxella bovis,* mas *M. ovis* e *M. bovoculi* são comumente isoladas de animais com CIB. As medidas terapêuticas disponíveis para o seu controle têm limitado sucesso e geram altos custos, justificando a importância da busca de novas alternativas de tratamento. A terapia fotodinâmica é um método que utiliza moléculas fotoativas, como as porfirinas, que interagem com a luz gerando espécies reativas de oxigênio que causam morte celular. Assim, essa dissertação teve por objetivo avaliar a atividade antibacteriana de metaloporfirinas tetra-catiônicas (**H_2TMeP** e **ZnTMeP**) frente a isolados e cepas padrão de *Moraxella* spp., *in vitro*, em um modelo *ex vivo* e sobre células sésseis. No experimento 1 (capítulo 1) foi avaliada a atividade antibacteriana das porfirinas frente a células planctônicas de *Moraxella* spp. *in vitro* e *ex vivo*. Para a análise *in vitro*, cada porfirina (4,0 μM) foi incubada com ~1x10^4 unidades formadoras de colônia (UFC) por mL de cada isolado de *Moraxella* spp. (n=22). Esta solução (200 μL) foi exposta à luz artificial por 0; 2,5; 5,0 e 7,5 min, ou mantida no escuro (controle) e, em seguida, plaqueada e incubada por 24 h para quantificação de UFC. Todos os isolados clínicos e as cepas padrão de *Moraxella* spp. foram completamente inativados pela porfirina **ZnTMeP** em até 7,5 min de irradiação. **H_2TMeP** não apresentou atividade antibacteriana frente a *Moraxella* spp., por isso, os demais experimentos foram realizados apenas com a porfirina **ZnTMeP**. Para o ensaio *ex vivo*, córneas excisadas do globo ocular de bovinos abatidos foram irrigadas com uma cultura de *Moraxella* spp. (~1x10^4 UFC/mL) seguida da adição da porfirina. As peças foram irradiadas por 0; 7,5 e 30 min, ou mantidas no escuro. Suabes das córneas foram coletados e semeado para quantificação de UFC. A porfirina **ZnTMeP** promoveu redução significativa ($p<0,05$) na concentração das cepas de *Moraxella* spp. após 30 min de irradiação. No segundo experimento (capítulo 2) foi avaliada a atividade antibacteriana da porfirina **ZnTMeP** e do cloridrato de oxitetraciclina (OXY) frente a células sésseis de *M. bovis, M. bovoculi* e *M. ovis* (durante e após a consolidação do biofilme). **ZnTMe**P (4,0 μM) e OXY (20 μg/mL) foram utilizadas isoladamente e em associação durante e após a consolidação do biofilme. **ZnTMeP** não apresentou efeito em ambas as fases. OXY foi capaz de reduzir a formação de biofilmes de todas as cepas. Frente à biofilme consolidado, OXY reduziu o número de células viáveis de *M. bovoculi* e *M. ovis*, porém não alterou a viabilidade de *M. bovis* em biolfime consolidado. A combinação de **ZnTMe**P e OXY contra *Moraxella* spp. em biofilme não apresentou efeito superior na destriuição das células em comparação à aplicação de cada composto isolamente. Esses resultados encorajam a realização de futuros experimentos *in vivo* utilizando **ZnTMeP** com intuito de inativar células plantônicas de *Moraxella* spp. causadoras de IBK. Por outro lado, foi reiteramos que a busca ativa por novos compostos e terapias que tenham ação efetiva sobre biofilmes de *Moraxella* spp. é fundamental.

Palavras-chave: pinkeye; terapia fotodinâmica; células sésseis; metaloporfirinas; antibiótico

ABSTRACT

ACTIVITY OF TETRA-CATIONIC PORPHYRINS AGAINST ISOLATES AND BIOFILMS OF *Moraxella* spp. INVOLVED IN INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

AUTHOR: Marlane Geribone Seeger
ADVISOR: Juliana Felipetto Cargnelutti

Infectious keratoconjunctivitis (IBK) is the most important eye disease in ruminant worldwide and, although it is not fatal, it can cause large losses in animal production. The primary agent of the disease is *Moraxella bovis*, but *M. ovis* and *M. bovoculi* are commonly isolated from animals with IBK. The therapeutic measures available for its control have limited success and generate high costs, justifying the importance of searching for new treatment alternatives. Photodynamic therapy is a method that uses photoactive molecules, such as porphyrins, that interact with light generating reactive oxygen species that cause cell death. Thus, this dissertation aimed to evaluate the antibacterial activity of tetra-cationic metalloporphyrins (**H_2TMeP** and **ZnTMeP**) against isolates and reference strains of *Moraxella* spp., *in vitro*, in an *ex vivo* model and on sessile cells. In experiment 1 (chapter 1) the antibacterial activity of porphyrins against planktonic cells of *Moraxella* spp. *in vitro* and *ex vivo*. For *in vitro* analysis, each porphyrin (4.0 μM) was incubated with ~1x10^4 colony forming units (CFU) per mL of each *Moraxella* spp. (n=22). This solution (200 μL) was exposed to artificial light for 0; 2.5; 5.0 and 7.5 min, or kept in the dark (control) and then plated and incubated for 24 h for CFU quantification. All clinical isolates and reference strains of *Moraxella* spp. were completely inactivated by **ZnTMeP** porphyrin within 7.5 min of irradiation. **H_2TMeP** did not show antibacterial activity against *Moraxella* spp., therefore, the other experiments were performed only with the porphyrin **ZnTMeP**. For the *ex vivo* assay, corneas excised from the eyeballs of slaughtered cattle were irrigated with a culture of *Moraxella* spp. (~1x104 CFU/mL) followed by addition of porphyrin. The pieces were irradiated by 0; 7.5 and 30 min, or kept in the dark. Corneal swabs were collected and seeded for CFU quantification. **ZnTMeP** promoted a significant reduction ($p<0.05$) in the concentration of *Moraxella* spp. after 30 min of irradiation. In the second experiment (chapter 2) the antibacterial activity of porphyrin **ZnTMeP** and oxytetracycline hydrochloride (OXY) against sessile cells of *M. bovis*, *M. bovoculi* and *M. ovis* (during and after the consolidation of the biofilm) was evaluated. **ZnTMeP** (4.0 μM) and OXY (20 μg/mL) were used alone and in association during and after biofilm consolidation. **ZnTMeP** had no effect in both phases. OXY was able to reduce biofilm formation of all strains. Against consolidated biofilm, OXY reduced the number of viable cells of *M. bovoculi* and *M. ovis*, but did not change the viability of *M. bovis* in consolidated biofilm. The combination of **ZnTMeP** and OXY against *Moraxella* spp. in biofilm showed no superior effect on cell destruction compared to the application of each compound alone. These results encourage the performance of future *in vivo* experiments using **ZnTMeP** in order to inactivate planktonic cells of *Moraxella* spp. causing IBK. On the other hand, we reiterate that the active search for new compounds and therapies that have an effective action on *Moraxella* spp. it's fundamental.

Key words: pinkeye; photodynamic therapy; sessile cells; antibiotic

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1 INTRODUÇÃO

A ceratoconjuntivite infecciosa bovina (CIB) é a doença ocular mais relevante na criação dos ruminantes em todo o mundo, podendo afetar até 80% dos rebanhos. A enfermidade ocorre geralmente na forma de surtos e causa grandes prejuízos econômicos (POSTMA et al., 2008). O agente primário da doença é a *Moraxella bovis* (ANGELOS, 2015), mas *Moraxella ovis* e *Moraxella bovoculi* são comumente isolados de animais com sinais de CIB (ELAD et al., 1988; ANGELOS et al., 2007). As cepas patogênicas de *M. bovis* possuem fatores de virulência necessários para o estabelecimento da doença, tais como a expressão de fímbrias tipo IV e a secreção de citotoxina com propriedades hemolítica e leucocítica (PUGH; HUGHES, 1968; BEARD; MOORE, 1994). Além disso, estudos comprovaram que isolados de *M. bovis, M. ovis* e *M. bovoculi* são capazes de formar biofilmes (PRIETO et al., 2013; ELY et al., 2018).

O impacto econômico da doença é decorrente das lesões oculares, que são responsáveis pela da perda da visão, resultando em diminuição do ganho de peso, redução na produção de leite, dificuldades de manejo dos animais e elevados custos com tratamentos (SLATTER et al., 1982; CONCEIÇÃO; TURNES, 2003).

Os animais afetados podem apresentar lacrimejamento, conjuntivite, blefaroespasmo, fotofobia, edema de córnea associado ao aparecimento de um foco opaco no centro da córnea, progredindo em casos mais graves para ulceração corneal, ruptura de córnea e prolapso de íris, resultando em cegueira permanente (CARMO et al., 2011). Desta forma, o impacto econômico decorre de lesões oculares, que são responsáveis pela da perda da visão, resultando em diminuição do ganho de peso, redução na produção de leite, dificuldades de manejo dos animais e elevados custos com tratamentos (SLATTER et al., 1982; CONCEIÇÃO; TURNES, 2003).

O tratamento para essa enfermidade consiste controle da multiplicação bacteriana na mucosa ocular, principalmente com o uso de antimicrobianos. Uma vez que a terapia antimicrobiana não garante a erradicação do estado de animal portador da enfermidade (BROWN et al., 1998), é comum observar o reaparecimento da doença em animais previamente submetidos ao tratamento (ANGELOS et al., 2011). As medidas terapêuticas disponíveis para controlar a CIB têm limitado sucesso e geram altos custos para a produção, principalmente devido à necessidade de múltiplas aplicações de antimicrobianos e assistência veterinária (MCCONNEL et al., 2007). O tratamento dos casos clínicos deve começar imediatamente após o diagnóstico da doença, como forma de impedir que ocorram lesões irreparáveis de córnea (KNEIPP, 2021).

Ainda, a formação de biofilmes é um processo clinicamente importante, pois essas comunidades bacterianas são de difícil eliminação, podem ser resistentes aos agentes antimicrobianos, e ainda tornar a infecção persistente no globo ocular dos animais (ANANE et al., 2020; CLUTTERBUCK et al., 2007; DONLAN, 2001; STEWART; COSTERTON, 2001). Neste cenário, é cada vez mais intensa a busca por métodos alternativos capazes de neutralizar e/ou eliminar os agentes infecciosos e seus respectivos biofilmes. Assim, a terapia fotodinâmica, através da utilização de porfirinas têm se destacado no combate às infecções bacterianas (BRANCO et al., 2018; SEEGER et al., 2020; TASLI et al., 2018).

A terapia fotodinâmica é um método aplicado às diversas áreas da saúde e tem como base a ação de um composto fotossensível. Estes compostos, quando ativado por diferentes comprimentos de onda de luz, produzem espécies reativas de oxigênio (ROS) que podem ser radicais livres e/ou oxigênio singleto ($^1O_2$), capazes de induzir a morte celular (MACHADO, 2000). Além disso, estes foto-oxidam as proteínas, lipídeos e DNA, inativando ou alterando diversas moléculas celulares (CASTEEL et al., 2004). Existem diversos compostos fotossensíveis que vêm sendo utilizados em medicina humana e animal. Destacam-se as porfirinas, que são compostos macrociclos tetrapirrólicos composto de quatro anéis heterocíclicos (A, B, C, D) ligados entre si por grupos de meteno (-CH=), aleatoriamente denominados α, β, γ, δ (RIMINGTON & KENNEDY, 1962). As porfirinas podem estar associadas aos íons metálicos, sendo chamadas de metaloporfirinas, e quando irradiadas pela luz, absorvem energia e geram espécies reativas de oxigênio (ALVES, 2014).

A terapia fotodinâmica vem sendo reconhecida *in vitro* e *in vivo* como uma alternativa muito promissora ao tratamento com antimicrobianos, especialmente em infecções localizadas (ALMEIDA et al., 2014), como casos de otite canina por *Pseudomonas aeruginosa*(SELLERA et al., 2019). Suas principais vantagens são a capacidade de inativar células bacterianas sem induzir à resistência, devido ao modo de ação e ao tipo de alvos bioquímicos, e o fato de não afetar a microbiota de outros sistemas do paciente, já que a irradiação ocorre de forma localizada (TAVARES et al., 2010).

Assim, esta dissertação teve como objetivo contribuir para o desenvolvimento de métodos de inativação de *Moraxella* spp. sensíveis e/ou resistentes aos antimicrobianos tradicionais e formadoras de biofilme, através da terapia fotodinâmica utilizando porfirinas tetracatiônicas. Foram produzidos dois manuscritos que estão apresentados no formato artigo científico.

2 REVISÃO BIBLIOGRÁFICA

2.1 CERATOCONJUNTIVITE INFECCIOSA BOVINA

A ceratoconjuntivite infecciosa bovina (CIB, "pinkeye") é a doença ocular mais relevante na criação de ruminantes em todo o mundo e, embora não seja comumente fatal, pode causar grandes perdas econômicas na produção desses animais (POSTMA et al., 2008). A CIB é uma enfermidade altamente contagiosa e de distribuição mundial, geralmente com prevalência elevada. Normalmente, ocorre na forma de surtos, podendo atingir até 80% do rebanho e perdurando por 3 a 4 semanas (POSTMA et al., 2008).

Embora *Moraxella bovis* seja o agente primário da doença (ANGELOS, 2015), *Moraxella ovis* e *Moraxella bovoculi* são frequentemente isoladas de animais com CIB (ELAD et al., 1988; ANGELOS et al., 2007). O gênero *Moraxella* compreende bactérias Gram-negativas, imóveis, aeróbicas, oxidase positiva, variáveis para catalase e colagenase, que não esporulam, não fermentam carboidratos, nem reduzem nitratos. O pleomorfismo é característico da espécie, apresentando-se como cocos ou aos pares, como *M. ovis* e *M. bovoculi*, ou na forma de cocobacilos e bacilos em cadeias curtas, como *M. bovis* (QUINN et al., 2005).

As cepas patogênicas de *M. bovis* possuem fatores de virulência necessários para o estabelecimento da doença, tais como a expressão de fímbrias tipo IV e a secreção de citotoxina com propriedades hemolítica e leucocítica (PUGH; HUGHES, 1968; BEARD; MOORE, 1994). Prieto et al. (2013) demonstrou a formação de biofilmes em isolados de *M. bovis* que apresentavam pili tipo IV. Corroborando com esses estudos, Ely et al. (2018) comprovou a capacidade de formação de biofilmes por isolados de *M. bovis, M. ovis* e *M. bovoculi*.

As taxas de prevalência da doença podem variar por região geográfica, clima, idade e tamanho do rebanho, tornando muitas vezes difícil generalizar a ocorrência da enfermidade em um período determinado. Além disso, poucos países têm realizado estudos de prevalência nacional da CIB, tornando essa, uma das principais doenças bovinas com prevalência e/ou ocorrência subestimadas (DENNIS; KNEIPP, 2021)

De acordo com os estudos publicados, a prevalência da doença é muito variável. Há relatos de até 45% dos animais afetados durante o verão em alguns rebanhos nos Estados Unidos (ARORA et al., 1976). Por outro lado, um levantamento feito em bezerros no mesmo país demonstrou que a prevalência foi de 6,5% (SNOWDER et al., 2005). Na Austrália, uma pesquisa recente com produtores de carne bovina, indicou que 94,1% dos animais tiveram CIB

entre 2014 e 2018, e 35,5% relataram a ocorrência da enfermidade durante todos os anos no período avaliado (KNEIPP et al., 2021b).

O impacto econômico da doença é decorrente das lesões oculares, que são responsáveis pela perda da visão, resultando em diminuição do ganho de peso, redução na produção de leite, dificuldades de manejo dos animais e elevados custos com tratamentos (SLATTER et al., 1982; CONCEIÇÃO; TURNES, 2003). Em 2006, a doença custou $23,2 milhões à indústria de carne bovina australiana (SACKETT et al., 2006) e $13,3 milhões em 2015 (LANE et al., 2015). Kneipp et al. (2021a) avaliaram que produtores australianos gastaram anualmente US $9,7 milhões no período de 2015 a 2018, apenas em três medicamentos comumente usados no tratamento da CIB.

A CIB é uma enfermidade altamente contagiosa que pode ser transmitida por contato direto, descarga ocular ou nasal de animais portadores e através de vetores mecânicos (*Musca autumnalis* e *Musca domestica*) que carreiam o agente. *M. bovis* pode sobreviver por mais de três dias nas patas das moscas (GERHARDT et al., 1982). A raça dos animais, a idade e o clima geográfico foram identificados como os fatores de risco mais importantes para a ocorrência da CIB (DENNIS; KNEIPP, 2021). Conceição & Turnes (2003), afirmam que em estabelecimentos onde a doença é endêmica, as taxas de incidência são maiores nos animais jovens.

Animais pertencentes a espécie *Bos indicus*, são mais adaptados a climas quentes, exibem maior resistência a carrapatos, parasitas internos, estresse por calor e doenças oculares, incluindo a CIB (COOKE et al., 2020). Assim, em relação às raças de bovinos, zebuínos e suas cruzas são menos frequentemente afetados (WEBBER; SELBY, 1981). *Bos taurus*, são animais adaptados a climas mais frios, são raças de gado originárias da Europa e mais propensas a CIB, como as raças Hereford e Aberdeen Angus (SNOWDER et al., 2005; COOKE et al., 2020). A maior prevalência de CIB em Hereford está associada a fatores como a falta de pigmentação da pálpebra e a ineficiente ação bactericida da lágrima (SNOWDER et al., 2005).

Os sinais clínicos observados em animais afetados são lacrimejamento, conjuntivite, blefaroespasmo, fotofobia, dor ocular, progredindo para edema e opacidade de córnea associado ao aparecimento de um foco opaco no centro da córnea, progredindo em casos mais graves para ulceração corneal, ruptura de córnea e prolapso de íris, resultando em cegueira permanente (CARMO et al., 2011; KNEIPP et al., 2021b). Animais seriamente afetados podem apresentar perda de apetite e perda de peso. O período de incubação pode variar entre 8 e 18 dias (BAPTISTA, 1979).

Em ovinos, a ceratoconjuntivite infecciosa é denominada de oftalmia contagiosa ovina, uma doença epizoótica aguda e contagiosa, de ocorrência condicionada a falhas no manejo sanitário, alta densidade populacional, causas naturais e climáticas, como poeira, traumas, presença de moscas e radiação solar (MARGATHO et al., 2006). Os agentes etiológicos primários da enfermidade são *Mycoplasma conjunctivae* e *Clamydophila psittaci* (ALMEIDA et al., 2004). *M. ovis* comporta-se como um agente oportunista, responsável pela severidade e agravamento da doença (DAGNALL, 1994).

O teste laboratorial mais utilizado para confirmar o diagnóstico de CIB é a cultura microbiológica a partir de suabes oculares. O microrganismo também pode ser isolado de animais saudáveis, uma vez que a conjuntiva e a nasofaringe de bovinos e ovinos são reservatórios destes patógenos (POSTMA et al., 2008). *Moraxella* spp. são lábeis e facilmente tem seu crescimento suprimido por outros microrganismos (CHANDLER et al., 1979; KNEIPP et al., 2021b). Ensaio de imunoabsorção enzimática (ELISA) e testes de anticorpos fluorescentes para anticorpos anti-*M. bovis* também são descritos (KNEIPP et al., 2021a). Técnicas moleculares, como reação em cadeia da polimerase (PCR) e análise de sequenciamento de nucleotídeos do RNA ribossômico 16S são usados na identificação de *M. bovis* (O'CONNOR et al., 2012).

O tratamento para essa enfermidade consiste na eliminação da bactéria da mucosa ocular, porém muitas vezes isso não é possível, tornando recorrente o aparecimento da doença. Antimicrobianos são utilizados mundialmente para o tratamento de casos clínicos de CIB, porém ainda existem poucos estudos farmacológicos com os antibióticos comumente usados para o tratamento da enfermidade (O'CONNOR et al., 2021). O tratamento compreende a aplicação de antimicrobianos por via sistêmica, tópica e/ou subconjuntival (ANGELOS et al., 2011).

As medidas terapêuticas disponíveis para controlar a CIB têm limitado sucesso e geram altos custos para a produção. A escolha do tratamento pode ser influenciada por alguns fatores, tais como a eficácia dos antimicrobianos, custo, exigência e disponibilidade de trabalho, qualidade das instalações e assistência veterinária (MCCONNEL et al., 2007). Além disso, deve-se levar em consideração a necessidade de combater duas ou mais espécies de *Moraxella* presentes na mesma lesão (CONCEIÇÃO; TURNES, 2003). Estudos recentes demonstraram que existem diferenças nos padrões de susceptibilidade entre cepas isoladas em locais distintos e de um mesmo rebanho, indicando a necessidade de determinar a sensibilidade *in vitro* antes de iniciar o tratamento (CONCEIÇÃO; TURNES, 2003). Contribuindo com esses fatos, a capacidade de formar biofilmes facilita a sobrevivência do microrganismo, uma vez que

bactérias em biofilmes podem ser até 1000 vezes menos sensíveis aos antibióticos do que as bactérias planctônicas (STEWART; COSTERTON, 2001).

A terapia antimicrobiana não garante a erradicação do estado de animal portador de *Moraxella* spp. (BROWN et al., 1998). O tratamento dos casos clínicos deve iniciar imediatamente após o diagnóstico da doença, como forma de impedir que ocorram lesões irreparáveis de córnea. Antimicrobianos injetáveis muitas vezes apresentam períodos de carência elevados, com risco de deixar resíduos no leite e na carne (ALEXANDER, 2010). Altas concentrações de antibiótico também podem ser atingidas no filme lacrimal por injeção subconjuntival, no entanto, essa forma de administração pode causar reações locais indesejáveis (CONCEIÇÃO; TURNES, 2003). O tratamento tópico é frequentemente impossibilitado pela dificuldade de manejo, uma vez que podem ser necessárias várias aplicações para que se atinja uma concentração de antimicrobianos suficiente e constante na conjuntiva ocular (MCCONNEL et al., 2007). Antibióticos em apresentação sprays quando pulverizados no olho podem ser irritantes e permanecem apenas alguns minutos no local, pois rapidamente são removidos pelas lágrimas (WARD; CLARK, 1991).

A resposta ao tratamento com antibióticos frequentemente usados para CIB diferem entre os isolados de regiões distintas. Conceição et al. (2004) comprovou que *M. bovis* possui padrões de suscetibilidade a antibióticos que variam entre os países e os períodos de recuperação, indicando a necessidade de determinar a eficácia do antibiótico de escolha antes de iniciar seu uso terapêutico Além disso, a eficácia terapêutica é afetada pela frequência e modo de administração dos fármacos; as possibilidades de manejo variam entre propriedades intensivas e extensivas, influenciando no resultado dos tratamentos aplicados (SEID, 2019).

M. bovis é frequentemente susceptível à gentamicina, cefalosporinas de primeira geração, trimetroprima-sulfonamidas, nitrofuranos e tetraciclinas (BROWN et al., 1998), aplicados topicamente em suspensão, pomadas ou aerossóis. Ademais, a via de administração deve ser considerada ao escolher um antibiótico para tratar a enfermidade. Os aminoglicosídeos não alcançam concentrações terapêuticas em lágrimas após a administração parenteral (FRASER, 1996).

Pesquisas recentes demonstraram que diferentes isolados de *Moraxella* spp. apresentam resistência aos principais antimicrobianos usados no tratamento da CIB, como penicilina G, oxitetraciclina e florfenicol (ANGELOS et al., 2011; MABONI et al., 2015), demonstrando a necessidade de se buscar novas alternativas para o tratamento da CIB. Cepas de *M. bovoculi*, apresentando perfil de multirresistência à maioria dos antibióticos aprovados para tratamento da doença, já foram descritas nos Estados Unidos, sendo recentemente listadas na American

Veterinary Medical Association, como patógenos de interesse, resistentes a antimicrobianos que afetam a saúde animal (AVMA, 2020; O'CONNOR et al., 2021).

O controle e a profilaxia da CIB também inclui o uso de vacinas. No entanto, as vacinas existentes possuem uma baixa eficácia (O'CONNOR et al., 2019). Essa situação pode ser explicada pela variação genética e antigênica entre as cepas de *M. bovis* e a presumida presença de outros microrganismos envolvidos na ocorrência da doença (CONCEIÇÃO; TURNES, 2003; KOWALSKI et al., 2017).

2.2 TERAPIA FOTODINÂMICA

A terapia fotodinâmica tem como base a ação de um composto fotossensível que quando ativado por diferentes comprimentos de onda de luz, produz espécies reativas de oxigênio (EROS) que são capazes de induzir a apoptose das células (MACHADO, 2000) ou foto-oxidar proteínas, lipídeos e ácidos nucleicos, inativando ou alterando diversas moléculas celulares (CASTEEL et al., 2004). É um método aplicado às diversas áreas da saúde, podendo ser usado no tratamento de tumores, no diagnóstico ou profilaxia pré e pós-cirúrgica, na degeneração macular da retina, psoríase, arteriosclerose, otites (SIMPLÍCIO et al., 2002; DAI et al., 2009; SELLERA et al., 2019). Além disso, é reconhecida pela sua eficácia contra vários microrganismos, incluindo vírus (BASSO et al., 2019; RIES et al., 2020), bactérias (MERCHAT et al., 1996; SIMÕES et al., 2016; MARCIEL et al., 2018; AMOS-TAUTUA et al., 2019; MAMONE et al., 2016; SEEGER et al., 2020) e fungos (CORMICK et al., 2009).

Este tipo de terapia pode ser classificada em duas categorias de acordo com o método de aplicação: terapia direta, sem a administração de um fotossensibilizador, sendo realizada apenas pela ação de uma fonte de luz (BONNETT, 2000) e a terapia indireta, que envolve dois componentes não tóxicos (o fotossensibilizador e o oxigênio molecular) e uma fonte de luz. No caso da terapia indireta, o composto fotossensibilizador é administrado, geralmente, diretamente na lesão e, em seguida é irradiado para gerar as EROS que irão interagir com moléculas orgânicas, alterando estruturas e/ou funções celulares que resultam na morte e/ou inativação celular (BECHET et al., 2008).

A luz visível que é utilizada para excitar o fotossensibilizador tem comprimentos de onda específicos que coincidem com o comprimento de onda de absorção máxima do fotossensibilizador (AMOS-TAUTUA et al., 2019). Assim, quando o composto é ativado, ele transfere energia da luz para o oxigênio molecular resultando na formação de EROS que destroem as células-alvo (ALVES, 2014). As reações ocorrem no local em que o

fotossensibilizador absorve a luz, assim as respostas biológicas ao fotossensibilizador ocorrem apenas nas áreas em que o tecido foi exposto à irradiação (DOLMANS et al., 2003).

A inativação fotodinâmica baseia-se na produção de EROS, que podem ser radicais livres e/ou oxigênio singleto ($^1O_2$). Nas reações, o fotossensibilizador é ativado para um estado singleto excitado (S1). As moléculas nesse estado decaem rapidamente de volta ao estado fundamental (S0), com a emissão de luz (fluorescência) ou calor, ou para o estado tripleto (T1). O fotossensibilizador, no estado tripleto excitado (T1), pode decair para o estado fundamental por fosforescência ou agir nas seguintes reações: reação do tipo I, onde o fotossesibilizador excitado no estado tripleto (T1) reage diretamente com o substrato ($^3O_2$), formando EROS, tais como os ânions superóxido, radicais hidroxil e peróxido de hidrogênio; na reação do tipo II, o fotossensibilizador excitado no estado tripleto (T1), transfere energia diretamente para o oxigênio molecular oxigênio molecular ($^3O_2$), originando oxigênio singleto ($^1O_2$). O oxigênio singleto é extremamente reativo podendo interagir com um grande número de substratos biológicos, induzindo, assim, dano oxidativo e morte celular (ALVES, 2014).

Entre os fotossensibilizadores que podem ser utilizados na terapia fotodinâmica destacam-se as porfirinas, que são compostos macrociclos tetrapirrólicos constituídos de 4 anéis heterocíclicos (A, B, C, D) ligados entre si por grupos de meteno (-CH=), aleatoriamente denominados α, β, γ, δ (RIMINGTON; KENNEDY, 1962). Podem estar associadas aos íons metálicos, sendo chamadas de metaloporfirinas (SOBOTTA et al., 2019). A quelação de um metal por um anel de porfirina pode aumentar o cruzamento intersistema, potencialmente levando ao aumento da geração de espécies reativas de oxigênio, principalmente espécies $^1O_2$ (HARRIMAN et al., 1983; SKWOR et al, 2016).

As porfirinas catiônicas são mais eficientes contra bactérias, principalmente Gram-negativas, em comparação às aniônicas ou neutras (MERCHAT et al., 1996; SIMÕES et al., 2016; MARCIEL et al., 2018; AMOS-TAUTUA et al., 2019). As porfirinas catiônicas podem atuar contra os dois tipos de bactérias, provavelmente devido a uma ação dupla: rompimento da parede celular bacteriana e, posteriormente, fotossensibilização da célula (CARVALHO et al., 2007).

As bactérias Gram-positivas e Gram-negativas possuem diferenças fundamentais em sua estrutura e, consequentemente, na sensibilidade aos efeitos dos fármacos. A parede celular das bactérias Gram-positivas tem um grau de porosidade relativamente alto. Portanto, várias macromoléculas como glicopeptídeos e polissacarídeos, com peso molecular de até 60 kDa podem facilmente se difundir através da parede celular. Dessa forma, não é uma barreira para

a penetração da maioria dos fotossensibilizadores, cujo peso molecular geralmente não excede 1,5-1,8 kDa (MEEROVICH et al., 2018).

A parede celular das bactérias Gram-negativas tem um elemento estrutural adicional, a membrana externa, que possui 10-15nm de espessura, localizando-se externamente à rede de peptidoglicano. Tem uma composição muito heterogênea, com proteínas com função de porina, trímeros de lipopolissacarídeos e lipoproteínas que criam uma pseudo-superfície externa de cargas negativas fortemente compactadas. Esse sistema altamente organizado impede a penetração de grandes moléculas e facilita a resistência aos diferentes compostos químicos. Compostos hidrofílicos com uma massa molecular abaixo de 0,6-0,7 kDa podem se difundir facilmente através dos poros. Somente um fotossensibilizador policatiônico com um tamanho de molécula e peso molecular bastante pequeno interage efetivamente com bactérias Gram-negativas, inativando-as. Para garantir a inativação adequada das bactérias, é necessário usar altas concentrações de fotossensibilizadores durante a sensibilização (MEEROVICH et al., 2018).

A terapia fotodinâmica antimicrobiana vem sendo reconhecida como uma alternativa muito promissora ao tratamento com antimicrobianos, especialmente em infecções localizadas (ALMEIDA et al., 2014; SELLERA et al., 2019). O efeito dessa terapia é capaz de efetivamente inativar células bacterianas sem desenvolver resistência em resposta ao tratamento e, graças à irradiação local, não afeta a microflora de outros sistemas do paciente, ao contrário da maioria dos fármacos antimicrobianos (MEEROVICH et al., 2018). A principal vantagem desta tecnologia sobre os fármacos antimicrobianos é a ação multialvo (ALVES et al., 2015).

Desta forma, a terapia fotodinâmica pode ser um auxiliar no combate aos patógenos bacterianos resistentes, potencializando o efeito e/ou a eficácia antimicrobiana, ou ainda sendo utilizado como única forma de tratamento às infecções bacterianas superficiais.

3 CAPÍTULO 1

Antimicrobial efficacy of *in vitro* and *ex vivo* photodynamic therapy using porphyrins against bovine keratoconjunctivitis-causing *Moraxella* spp.

M.G. Seeger[1], C.S. Machado[1], B.A. Iglesias[2], F.S.F. Vogel[3], J.F. Cargnelutti[3*]

[1] Programa de Pós-Graduação em Medicina Veterinária, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil. [2] Laboratório de Bioinorgânica e Materiais Porfirínicos, Departamento de Química, UFSM, Santa Maria, RS, Brazil. [3] Departamento de Medicina Veterinária Preventiva (DMVP), Centro de Ciências Rurais (CCR), UFSM, Santa Maria, RS, Brazil. *Corresponding author: jucargnelutti@gmail.com

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Abstract

Infectious bovine keratoconjunctivitis (IBK) is an ocular disease affecting bovine herds worldwide, and it causes significant economic loss. *Moraxella bovis* is recognized asthe etiologic agent of IBK but *M. ovis* and *M. bovoculi* are frequently recovered of animals presenting clinical signs of IBK. The therapeutic measures available for its control have limited efficacy. Antimicrobial photodynamic therapy (aPDT) using porphyrins as photosensitizing molecules is an alternative method that can be used to reduce microbial growth. We evaluated the antibacterial activity of aPDT using two water-soluble tetra-cationic porphyrins (**H_2TMeP** and **ZnTMeP**) against 22 clinical isolates and standard strains of *Moraxella* spp. *in vitro* and in an *ex vivo* model. For the *in vitro* assay, 4.0 μM of porphyrin was incubated with approximately 1.0×10^4 CFU/mL of each *Moraxella* sp. isolate and exposed to artificial light for 0, 2.5, 5, and 7.5 min. Next, 50 μL of this solution was plated and incubated for 24 h until CFU measurement. For the *ex vivo* assay, corneas excised from the eyeballs of slaughtered cattle were irrigated with *Moraxella* spp. culture, followed by the addition of zinc (II) porphyrin **ZnTMeP** (4.0 μM). The corneal samples were irradiated for 0, 7.5, and 30 min, followed by swab collection, plating, and CFU count. The results demonstrated the *in vitro* inactivation of the strains and clinical isolates of *Moraxella* spp. after 2.5 min of irradiation using **ZnTMeP**, reaching complete inactivation until 7.5 min. In the *ex vivo* experiment, the use of **ZnTMeP** resulted in the most significant reduction in bacterial concentration after 30 min of irradiation. These results encourage future *in vivo* experiments to investigate the role of metalloporphyrin **ZnTMeP** in the inactivation of *Moraxella* spp. causing IBK.

Keywords: aPDT; cationic porphyrins; *Moraxella*; photoinactivation; pink eye.

Introduction

Infectious bovine keratoconjunctivitis (IBK) is the most common ocular disease in cattle, accounting for up to 80% of the infections (Postma et al. 2008). The disease has a substantial economic impact, including treatment cost, weight loss, and reduction in milk production. It also affects animal welfare by causing pain and blindness (Conceição and Turnes 2003; Dewell et al. 2014).

IBK affects animals of all ages regardless of sex. In a livestock where the disease is endemic, incidence rates are higher in calves and confined animals, than other categories (Gil-Turnes 2007; Fonseca et al. 2020). *Moraxella bovis* is recognized as the etiologic agent of IBK (Angelos 2015), but *Moraxella ovis* and *Moraxella bovoculi* are frequently recovered of animals presenting clinical signs of IBK (Elad et al. 1988; Angelos et al. 2007). *Moraxella* spp. are opportunistic pathogens occasionally found in the nasal and conjunctival secretions of cattle with or without clinical signs or history of disease (Markey et al. 2013). In affected animals, infection can result in corneal ulceration leading to blindness (Angelos 2015).

The therapeutic measures available to control IBK are costly and have limited success rates. The treatment regimen includes the administration of antimicrobial drugs through systemic, topical, or subconjunctival routes, depending on the drug to be used (Angelos 2015); however, antimicrobial therapy does not guarantee eradication of the bacteria (Brown et al. 1998). Some studies have demonstrated *Moraxella* spp. resistant against many antibiotics used for the treatment of IBK, such as penicillin G, oxytetracycline, and florfenicol (Angelos et al.

2011; Maboni et al. 2015). In addition, therapeutic efficacy is affected by the frequency and mode of drug administration, which influences treatment results (Seid 2019). Therefore, alternative methods for the treatment of IBK need to be investigated.

Photodynamic therapy (PDT) may be a promising alternative treatment. This therapy is used to treat tumors, arteriosclerosis, herpes lesions, and bacterial infections in humans (Simplício et al. 2002; Grammatikova et al. 2019; La Selva et al. 2020). In addition, antimicrobial photodynamic therapy (aPDT) can be used to inactivate microorganisms in solutions, fluids, and surfaces (Carvalho et al. 2007; Ries et al. 2020; Seeger et al. 2020). This method is able to inactivate bacterial cells without inducing resistance or affecting the normal microflora of the patient, unlike standard systemic administration drugs (Tavares et al., 2010).

Porphyrins are photosensitive compounds used in the photodynamic process. They are activated by a specific wavelength of light and produce reactive oxygen species (ROS) (Amos-Tautua et al. 2019). ROS can photo-oxidize proteins, lipids, and DNA, induce cell apoptosis, and inactivate or modify several molecules in cell (Machado 2000; Casteel et al. 2004). Cationic porphyrins are more efficient for photo-inactivation of bacteria, especially Gram-negative ones, than anionic or neutral porphyrins (Simões et al. 2016; Amos-Tautua et al. 2019). Studies have also revealed that cationic porphyrins act against bacteria by disrupting the bacterial cell wall, leading to subsequent photosensitization (Carvalho et al. 2007). Therefore, this study aimed to evaluate the *in vitro* and *ex vivo* antimicrobial activity of two water-soluble tetra-cationic porphyrins (free-base **H_2TMeP** and zinc (II) derivative [**ZnTMeP**]) against *Moraxella* spp.

Material and methods

Experimental design

The two tetra-cationic porphyrins, water-soluble *meso*-tetra-cationic free-base porphyrin (**H_2TMeP**) and its respective zinc (II) derivative (**ZnTMeP**) (Fig. 1), were purchased from Frontier Scientific® (Logan, UT, USA). The bacterial isolates were recovered from bovine samples sent for bacteriological diagnostic tests at the Bacteriology Laboratory of the Federal University of Santa Maria (LABAC/UFSM) (Table 1). All isolates were registered in SisGen (registration number A6E72A6), and the species were confirmed by PCR and restriction endonuclease analysis (Angelos & Ball 2007).

PLEASE INSERT IN THIS PLACE THE TABLE 1.

The causative agents of IBK, *M. bovis*, *M. ovis*, and *M. bovoculi* and/or their respective reference strains (ATCC®, Manassas, VA, USA), were incubated with each porphyrin and exposed to white-light irradiation for different periods (0, 2.5, 5, 7.5, or 30 min) or kept in the dark (control). For *in vitro* and *ex vivo* tests, 4.0 μM of porphyrin was used, as it was non-toxic to cells of the bovine lineage (MDBK; Ries et al. 2020). The porphyrin solutions were added in the culture medium inoculated with the test strains. The antibacterial activity of the porphyrins was determined by counting the colony-forming units (CFU).

PLEASE INSERT IN THIS PLACE THE FIGURE 1.

Irradiation conditions

Photo-irradiation assays were performed using white-light LED array system irradiation at a fluence rate of 25 mW/cm^2 and total light dosage of 45 J/cm^2 at 30 min, according to the method described by Alves et al. (2015). The distance between the light source and solution or corneal samples containing bacteria and porphyrin was approximately 5.0 cm. All experiments were performed independently in quadruplicate.

In vitro antibacterial activity of porphyrin against *Moraxella* spp.

The IBK-causing agents that were selected for antibacterial porphyrin assays were obtained from routine laboratory tests. All the assays were performed using twenty-two clinical isolates from cattle with IBK and three American Type Culture Collection (ATCC®) strains, as follows: *M. bovis* (ATCC® 10900), *M. bovoculi* (ATCC® BAA1259), and *M. ovis* (ATCC® 19575). All isolates were lyophilized and stored at −20 °C. Before performing the antibacterial assays, isolates were plated on brain heart infusion (BHI) agar and incubated at 37 °C for 24 h. *In vitro* assays were based on the protocol described by Seeger et al. (2020) with modifications. The tests were performed using approximately 1.0 × 10^4 CFU/mL in suspension with agitation, together with 4.0 μM of each porphyrin, and then exposed to white-light irradiation for 0, 2.5, 5, and 7.5 min. After light exposure, the suspensions were added in petri dishes containing BHI agar and incubated at 37 °C for 24 h. Next, the CFUs were counted to determine the antibacterial activity of the porphyrins. All procedures were performed in quadruplicate, containing bacteria and porphyrin with and without light exposure, as well as positive (only bacteria) and negative (only medium and porphyrin) controls.

Antibacterial activity of porphyrin against *Moraxella* spp. in an *ex vivo* model

The *ex vivo* experiments using corneas excised from the eyeballs were based on the *ex vivo* eye model described previously by Kompella et al. (2006) and Branco et al. (2018) with a few modifications. Freshly excised bovine eyes were obtained from a local slaughterhouse, placed in a plastic container at ambient temperature, and transported to the laboratory to excise the corneas. The experiments were initiated within 2–3 h after sacrifice.

The assays were performed using bovine corneas irrigated with reference cultures of *M. bovis*, *M. ovis*, or *M. bovoculi* and incubated with **ZnTMeP**, since it showed better results during *in vitro* experiments. All assays were conducted in three groups (light treatment, dark treatment, and control) containing two corneas each (duplicate). For the light and dark treatment groups, the bovine corneas were washed thrice with 0.5% chlorhexidine followed by sterile phosphate-buffered saline (PBS) solution and placed in 6-well plates. Then, 300 μL of each *Moraxella* spp. culture (previously prepared in BHI culture medium with OD_{600nm}: 0.3) was distributed over the corneas using a pipette to obtain a density of approximately 1.0 × 10^4 CFU/mL. An aliquot of 300 μL of **ZnTMeP** porphyrin diluted in culture medium (final concentration of 4.0 μM) was distributed using a pipette over the corneas exposed to light and/or kept in the dark.

For the control group, a solution containing bacterial culture and BHI medium was distributed over the corneas using a pipette. The corneas were then exposed to artificial light for 7.5 min or 30 min. After each irradiation period, a sterile swab was used to collect the bacteria from each cornea and suspended in 1 mL of PBS. Only 50 μL of this suspension was inoculated (in duplicate) on BHI agar plates. The plates were incubated at 37

°C for 24 h. Bacterial cell viability was determined by CFU counts after treatment with porphyrins. Three independent experiments were conducted in duplicates for each bacterium.

Statistical analysis

In both experiments, the statistical significance of the reduction in bacterial CFU counts owing to photodynamic inactivation was determined by analysis of variance (ANOVA), followed by Tukey's multiple comparison test using GraphPad Prism 6.0. Statistical significance was set at $p < 0.05$.

Results

In vitro PDT was tested using **ZnTMeP** (4.0 μM), which showed statistically significant ($p < 0.05$) activity against all clinical isolates and standard bacterial strains of *Moraxella* spp. **ZnTMeP** promoted the complete bacterial inactivation of *M. bovis* (Fig. 2) and *M. ovis* (Fig. 3) after 5 min of white-light irradiation under *in vitro* testing; however, for *M. bovoculi* (Fig. 4), complete bacterial inactivation occurred until 7.5 min. **H_2TMeP** did not show antibacterial activity against any isolate of *Moraxella* spp. after 7.5 min of white-light exposure. Antibacterial assays using reference bacterial strains demonstrated similar results to those obtained with bacteria isolated from cattle with IBK (Fig. 5).

PLEASE INSERT IN THIS PLACE FIG. 2, 3, 4 AND 5.

Once the ability of **ZnTMeP** to inactivate *Moraxella* spp. was confirmed in the *in vitro* assays, it was used for the *ex vivo* experiment. We found a significant decrease in *M. bovis* concentration after 7.5 min of irradiation, and it decreased further after 30 min (Fig. 6). The concentration of *M. bovoculi* and *M. ovis* significantly decreased after 30 min of irradiation (Fig. 6). In the control group, with and without white-light irradiation, there were no significant change in bacterial concentration after 7.5 and 30 min (Fig. 6).

PLEASE INSERT IN THIS PLACE THE FIG. 6.

Discussion

IBK has a detrimental impact on animal productivity. The disease can cause inflammation, and in severe cases, it may lead to temporary or permanent blindness in cows (Angelos 2015). The therapeutic measures available to control IBK have limited success rates, and the choice of treatment is influenced by the effectiveness of antimicrobials, cost, demand, and availability of work and veterinary care (Mcconnel et al. 2007).

Recent studies have shown that there are differences in the susceptibility patterns in strains isolated from different locations and from the same herd, indicating the need to determine *in vitro* sensitivity before starting any treatment (Conceição and Turnes 2003). In addition, there may be a need to combat two or more species of *Moraxella* present in the same lesion (Conceição and Turnes 2003).

Thus, the present study aimed to investigate an alternative treatment method for IBK in animals. We evaluated the *in vitro* and *ex vivo* antimicrobial activity of two water-soluble tetra-cationic porphyrins (free-base **H_2TMeP** and metallo-derivative **ZnTMeP**) against *Moraxella* spp. aPDT studies have revealed interesting results in different fields of medicine, and the use of porphyrins as photosensitizers has been shown to significantly

inactivate microorganisms in solutions/fluids on surfaces (Ries et al. 2020; Seeger et al. 2020). The mechanism of action is based on the application of photosensitizers activated locally by light at an appropriate wavelength, resulting in the generation of ROS, which further trigger modifications of several biological molecules and lead to cell death eventually (Ergaieg et al. 2008).

According to our antibacterial assays, **ZnTMeP** was able to inactivate all the *Moraxella* spp. isolates. It showed high activity against all clinical and standard bacterial strains after 7.5 min of white-light exposure *in vitro*. Moreover, it could significantly decrease *M. bovis*, *M. ovis*, and *M. bovoculi* concentrations in an *ex vivo* assay after 30 min of exposure. Bacterial inactivation was not observed in any assay performed under dark conditions, demonstrating the necessity of white-light irradiation to photo-inactivate and reduce bacterial concentration (Simões et al. 2016).

In the present study, **H_2TMeP** did not exhibit antibacterial activity. According to Pavani et al. (2009), zinc (II) porphyrins are more efficient in photodynamic inactivation compared to free-base porphyrins derivatives because the presence of zinc (II) ions decreases mitochondrial binding and increases membrane hydrophobicity (Pavani et al. 2009). Chelation of a metal by porphyrin ring can enhance intersystem crossing, potentially leading to increased generation of ROS (Harriman et al. 1983; Skwor et al 2016). Furthermore, earlier studies have indicated that tetra-cationic porphyrins are more efficient than anionic or neutral porphyrins against bacteria (Simões et al. 2016; Amos-Tautua et al. 2019), such as *Moraxella* sp. Cationic porphyrins promote the binding of photosensitizer molecules at critical cellular sites, resulting in cell damage caused by exposure to light (Jori and Brown 2004).

Our results showed differences in bacterial inactivation between *in vitro* and *ex vivo* assays. While *in vitro* assay showed complete inactivation of all species of *Moraxella* by 7.5 min, the *ex vivo* assay resulted in incomplete inactivation after 30 min. This result could be explained by the fact that under *in vitro* assay conditions, bacteria and photosensitizer are in solution, allowing the molecules to bind to each bacterium surface, resulting in a higher ROS yield near the bacteria and therefore a higher antibacterial efficacy. Therefore, bacterial inactivation is improved *in vitro* as compared to *in vivo* conditions, where bacteria exist as agglomerates or biofilms (Maisch et al. 2007; Branco et al. 2018). In the *ex vivo* model, the attachment of porphyrins to individual bacteria is reduced; consequently, the incubation time necessary to inactivate bacteria is higher than in the *in vitro* assay. Previous studies have reported similar results when the antibacterial efficacy of a lead photosensitizer was evaluated against susceptible and methicillin-resistant strains of *Staphylococcus aureus* using an *ex vivo* porcine skin model (Maisch et al. 2007; Branco et al. 2018).

Moreover, when the photosensitizer is supported or incorporated into an insoluble material, the efficiency of aPDT is significantly reduced, as light penetration is more difficult in tissues than in solution, which leads to a reduction in ROS levels (Carvalho et al. 2010; Branco et al. 20018). According to Demidova and Hamblin (2005), the effect on microbial photoinactivation of Gram-positive and negative bacteria is dependent on the cell concentration and the amount of photosensitizer binding. These results demonstrate the importance of testing the efficacy of aPDT in bacterial inactivation *in vitro* and *ex vivo*. The *ex vivo* model allows us to modify and define the appropriate methodology, as it is closely related to actual cellular conditions. Additionally, it does not require the use of live animals and enables researchers to promote reduced animal usage in studies.

In this study, we tested the effect of two porphyrins against 22 clinical isolates of *Moraxella* spp. and showed that porphyrins act similarly against field isolates. In addition, the standard strains often behave differently

from field isolates; however, our *in vitro* results demonstrated that they were inactivated in a similar manner. Therefore, they can be used in inactivation assays without compromising the interpretation of the actual efficiency of porphyrins.

Based on our results, aPDT could be considered effective in treating animals with IBK. In both studies, the alternative treatment showed promising results and a decrease in bacterial concentration; however, more studies are required before *in vivo* application for evaluating possible corneal injuries. Additionally, electron microscopic studies should be conducted to verify any microlesions caused by porphyrin in bacteria and in the cornea.

Conclusion

In summary, zinc (II) derivative **ZnTMeP** could be considered a promising photosensitizer for the inactivation of *Moraxella* spp. isolated from herds with IBK outbreaks. The *ex vivo* model demonstrated that the photodynamic inhibition of microorganisms occurs at a prolonged exposure time in comparison to the *in vitro* time. Further studies are required to determine the antibacterial activity *in vivo* through the evaluation of photodynamic therapy in cattle with IBK.

Conflicts of interest

Authors declare that they have no conflicts of interest.

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Fig. 1. Representative structures of tetra-cationic porphyrins used in this study. For clarity, the counter-ions (Cl^-) are omitted. A: water-soluble *meso*-tetra-cationic free-base porphyrin (**H_2TMeP**). B: Zinc (II) derivative (**ZnTMeP**).

Fig. 2. Antimicrobial activity of water-soluble *meso*-tetra-cationic free-base porphyrin (**H_2TMeP**) and its respective zinc (II) derivative (**ZnTMeP**) (both at 4.0 μM) against clinical isolates of *Moraxella bovis*. * indicates significant statistical variation between dark and white-light treatment with free-base **H_2TMeP**. ** indicates significant statistical variation between dark and white-light treatment with **ZnTMeP**. Error bars indicate standard deviation of mean (SD). (p<0.05).

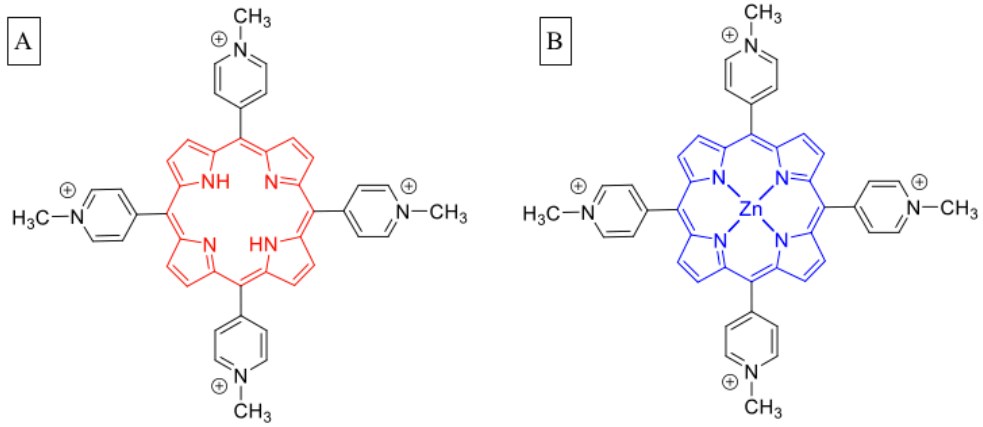
Fig. 3. Antimicrobial activity of water-soluble *meso*-tetra-cationic free-base porphyrin (**H_2TMeP**) and its respective zinc (II) derivative (**ZnTMeP**) (both at 4.0 μM) against clinical isolates of *Moraxella ovis*. * indicates significant statistical variation between dark and white-light treatment with free-base **H_2TMeP**. ** indicates significant statistical variation between dark and white-light treatment with **ZnTMeP**. Error bars indicate standard deviation of mean (SD). (p<0.05).

Fig. 4. Antimicrobial activity of water-soluble *meso*-tetra-cationic free-base porphyrin (**H_2TMeP**) and its respective zinc (II) derivative (**ZnTMeP**) (both at 4.0 μM) against clinical isolates of *Moraxella bovoculi*. * indicates significant statistical variation between dark and white-light treatment with free-base **H_2TMeP**. ** indicates significant statistical variation between dark and white-light treatment with **ZnTMeP**. Error bars indicate standard deviation of mean (SD). (p<0.05).

Fig. 5. Antimicrobial activity of water-soluble *meso*-tetra-cationic free-base porphyrin (**H_2TMeP**) and its respective zinc (II) derivative (**ZnTMeP**) (both at 4.0 μM) against standard bacterial strains of *Moraxella bovis* (ATCC® 10900), *Moraxella bovoculi* (ATCC® BAA1259), and *Moraxella ovis* (ATCC® 19575). * indicates significant statistical variation between dark and white-light treatment with free-base **H_2TMeP**. ** indicates significant statistical variation between dark and white-light treatment with **ZnTMeP**. Error bars indicate standard deviation of mean (SD). (p<0.05).

Fig. 6. Antimicrobial activity of water-soluble *meso*-tetra-cationic zinc (II) derivative **ZnTMeP** (4.0 μM) porphyrin against standard strains of *Moraxella bovis* (ATCC® 10900), *Moraxella bovoculi* (ATCC® BAA1259), and *Moraxella ovis* (ATCC® 19575) in an *ex vivo* model. The bars indicate standard deviation of mean (SD). **a**: indicates significant statistical variation between control and white-light treatment. **b**: indicates significant statistical variation between dark and white-light treatment with **ZnTMeP**. (p<0.05).

Fig. 1.



H_2TMeP **ZnTMeP**

Fig. 2.

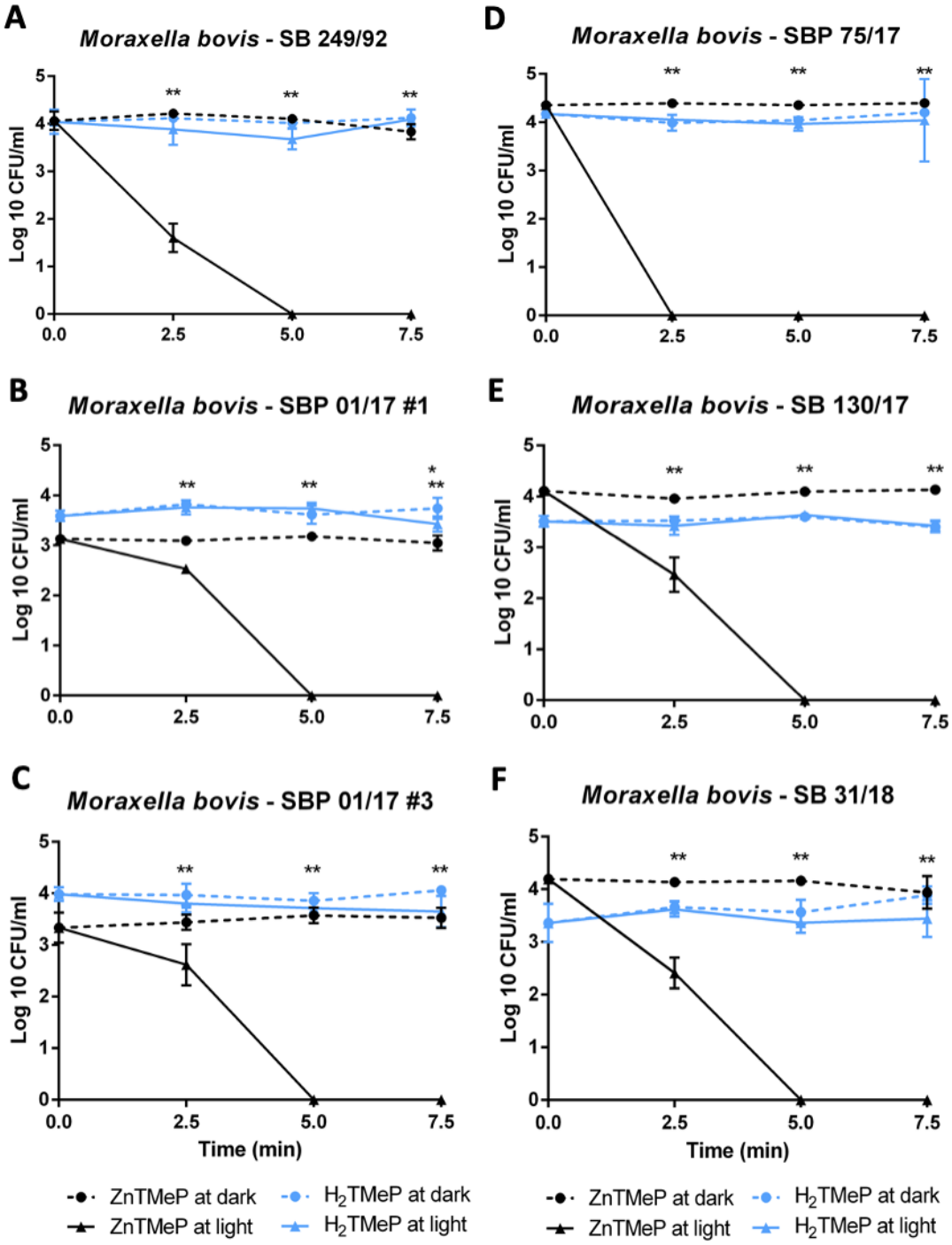

A
Moraxella bovis - SB 249/92
D
Moraxella bovis - SBP 75/17
B
Moraxella bovis - SBP 01/17 #1
E
Moraxella bovis - SB 130/17
C
Moraxella bovis - SBP 01/17 #3
F
Moraxella bovis - SB 31/18
Log 10 CFU/ml
Time (min)
ZnTMeP at dark
ZnTMeP at light
H2TMeP at dark
H2TMeP at light

Fig. 3.

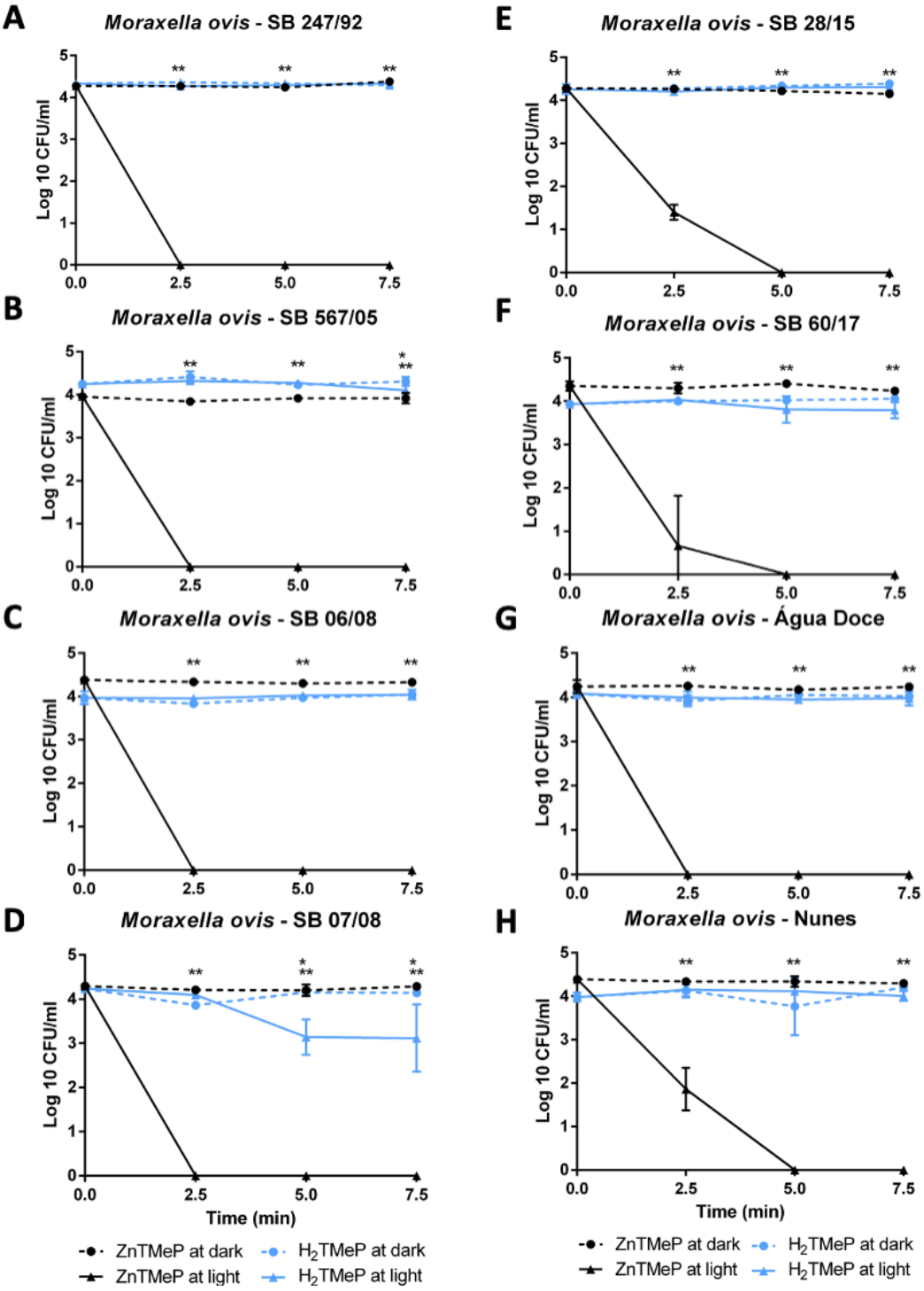

A
Moraxella ovis - SB 247/92
E
Moraxella ovis - SB 28/15
B
Moraxella ovis - SB 567/05
F
Moraxella ovis - SB 60/17
C
Moraxella ovis - SB 06/08
G
Moraxella ovis - Água Doce
D
Moraxella ovis - SB 07/08
H
Moraxella ovis - Nunes
Log 10 CFU/ml
Time (min)
ZnTMeP at dark
H2TMeP at dark
ZnTMeP at light
H2TMeP at light

Fig. 4.

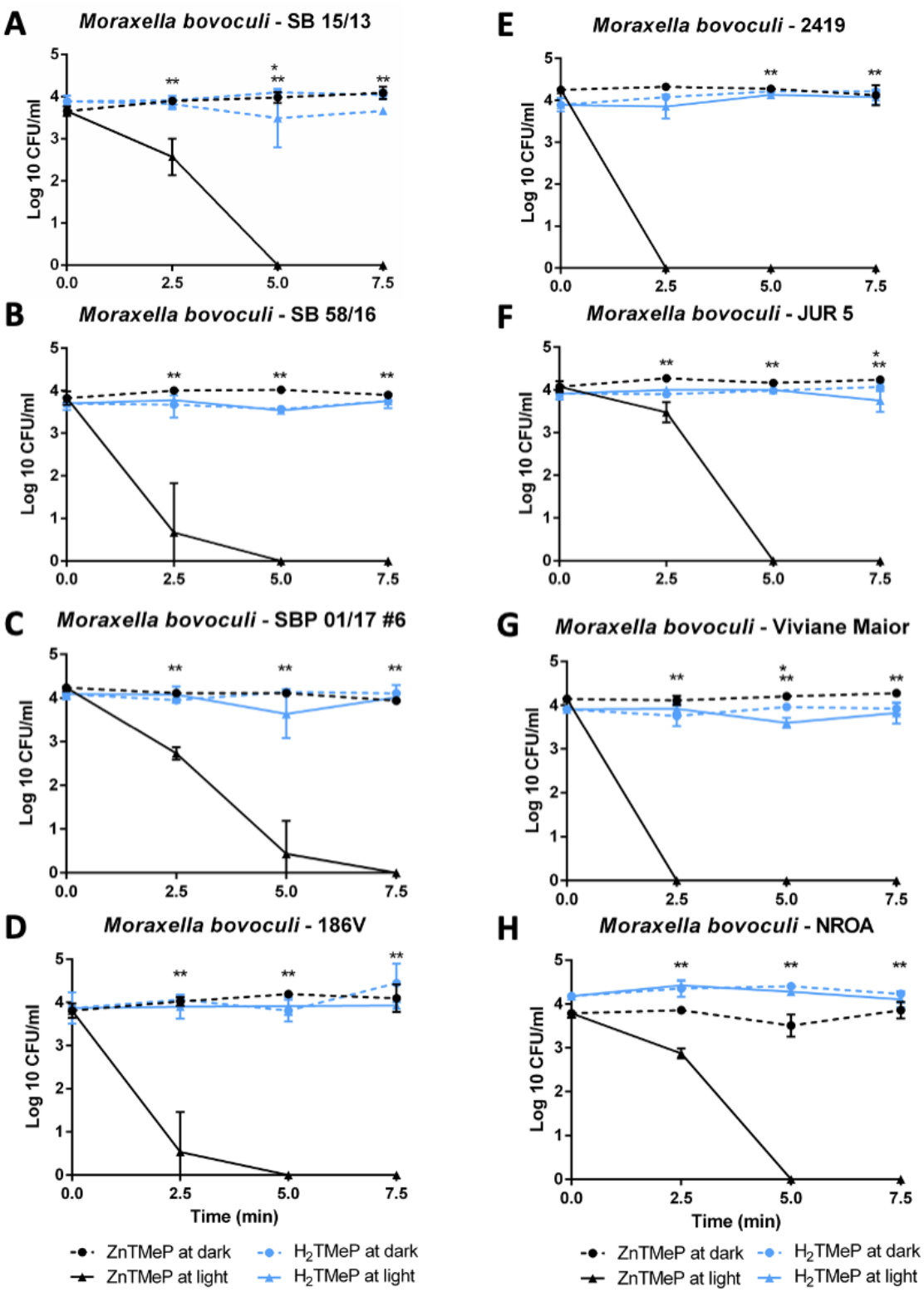

A
Moraxella bovoculi - SB 15/13
B
Moraxella bovoculi - SB 58/16
C
Moraxella bovoculi - SBP 01/17 #6
D
Moraxella bovoculi - 186V
E
Moraxella bovoculi - 2419
F
Moraxella bovoculi - JUR 5
G
Moraxella bovoculi - Viviane Maior
H
Moraxella bovoculi - NROA
Log 10 CFU/ml
Time (min)
ZnTMeP at dark
ZnTMeP at light
H2TMeP at dark
H2TMeP at light

Fig. 5.

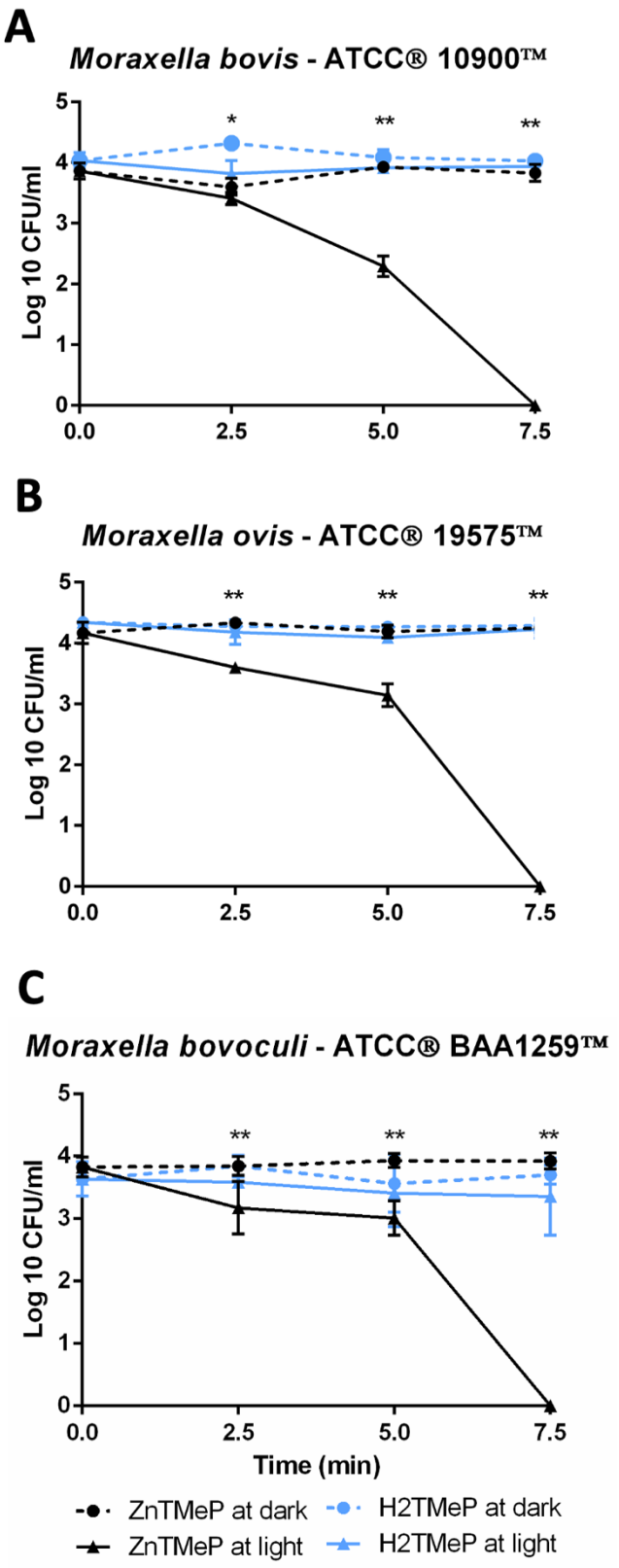

A
Moraxella bovis - ATCC® 10900™
Log 10 CFU/ml
0
1
2
3
4
5
0.0
2.5
5.0
7.5
*
**
**
B
Moraxella ovis - ATCC® 19575™
Log 10 CFU/ml
0
1
2
3
4
5
0.0
2.5
5.0
7.5
**
**
**
C
Moraxella bovoculi - ATCC® BAA1259™
Log 10 CFU/ml
0
1
2
3
4
5
0.0
2.5
5.0
7.5
Time (min)
**
**
**
ZnTMeP at dark
H2TMeP at dark
ZnTMeP at light
H2TMeP at light

Fig 6.

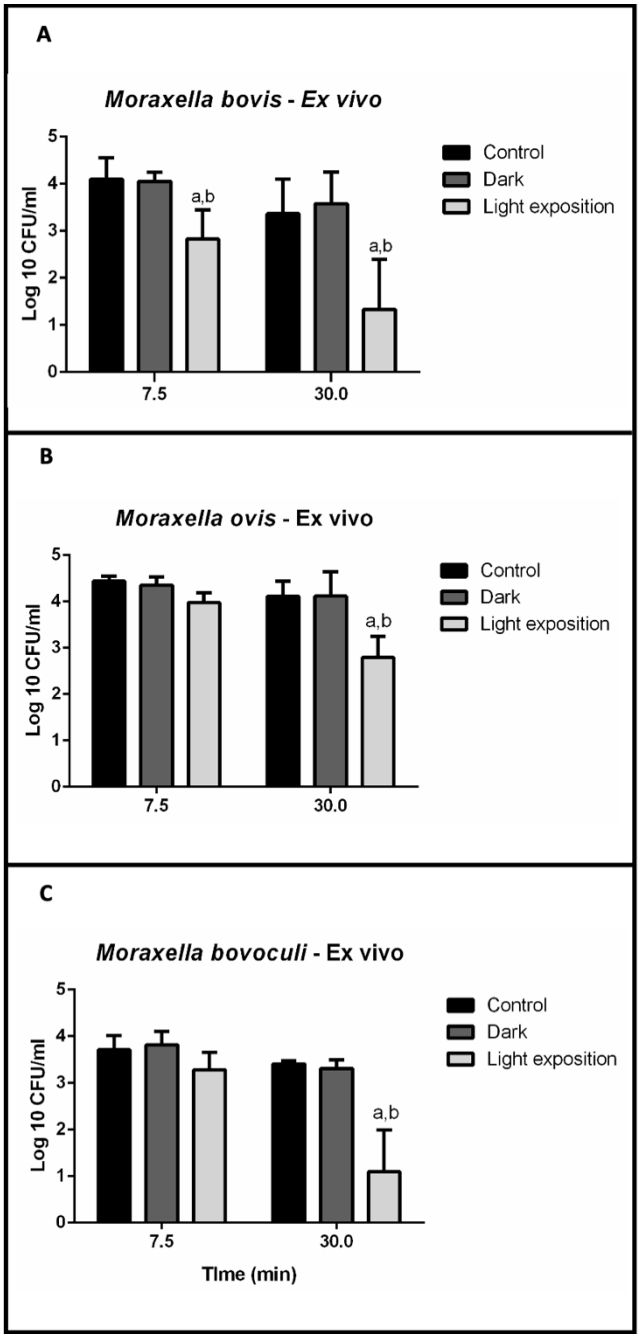

A
Moraxella bovis - Ex vivo
Log 10 CFU/ml
Control
Dark
Light exposition
a,b
a,b
7.5
30.0
B
Moraxella ovis - Ex vivo
Log 10 CFU/ml
Control
Dark
Light exposition
a,b
7.5
30.0
C
Moraxella bovoculi - Ex vivo
Log 10 CFU/ml
Control
Dark
Light exposition
a,b
7.5
30.0
Time (min)

Table 1 - Identification, origin, and year of isolation of *Moraxella bovis*, *M. bovoculi* and *M. ovis* strains used in antibacterial assays.

Specie	Identification	Origin	Year of isolation	GenBank ID
Moraxella bovis	SB 249/92	Cruz Alta/RS	1992	nd
	SBP 01/17 #1	Santa Maria/RS	2017	nd
	SBP 01/17 #3	Santa Maria/RS	2017	nd
	SBP 75/17	São Martinho da Serra/RS	2017	nd
	SB 130/17	Alegrete/RS	2017	nd
	SB 31/18	Tupanciretã/RS	2018	nd
Moraxella ovis	SB 247/92	Maringá/PR	1992	nd
	SB 567/05	Santa Maria/RS	2005	KP410775
	SB 06/08	Caçapava do Sul/RS	2008	KP410778
	SB 07/08	São Sepé/RS	2008	KP410779
	SBP 28/15	Dilermando de Aguiar/RS	2015	nd
	SB 60/17	Vila Nova do Sul/RS	2017	nd
	Água Doce	Belo Horizonte/MG	-	nd
	Nunes	Dom Pedrito/RS	1998	KP410774
Moraxella bovoculi	SB 15/13	Caçapava do Sul/RS	2013	nd
	SBP 58/16	-	2016	nd
	SBP 01/17 #6	Santa Maria/RS	2017	nd
	186V	Argentina	1999	KP410766
	2419	Florida - Uruguay	1983	DQ153089
	JUR 5	Pelotas/RS	2014	nd
	Viviane Maior	Pelotas/RS	2014	nd
	NROA	Pelotas/RS	2014	nd

(-) Information not available; nd: not deposited; RS: Rio Grande do Sul; MG: Minas Gerais.

1 CAPÍTULO 2

Biofilms of *Moraxella* spp. from cattle: activity of porphyrin and oxytetracycline alone and in combination

M.G. Seeger[1], B.A. Iglesias[2], F.S.F. Vogel[3], J.F. Cargnelutti[3*]

[1] Programa de Pós-Graduação em Medicina Veterinária, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil.

[2] Laboratório de Bioinorgânica e Materiais Porfirínicos, Departamento de Química, UFSM, Santa Maria, RS, Brazil.

[3]Departamento de Medicina Veterinária Preventiva (DMVP), Centro de Ciências Rurais (CCR), UFSM, Santa Maria, RS, Brazil.

*Corresponding author: jucargnelutti@gmail.com

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Abstract

Moraxella bovis, *Moraxella ovis* and *Moraxella bovoculi* are able to form biofilms and can be isolated from cattle presenting infectious bovine keratoconjunctivitis (IBK), the most important eye disease in ruminants worldwide. Oxytetracycline is frequently used as the first choice for antimicrobial treatment of affected cattle, but have limited success in clearing the infection. Photodynamic therapy using porphyrins as photosensitizing molecules is an alternative method that can be applied to eliminate microorganisms, including biofilms. Therefore, we evaluated the antibacterial activity of a zinc (II) porphyrin (**ZnTMeP**) and oxytetracycline hydrochloride (OXY) alone and in combination against biofilms of *Moraxella* spp. The minimal inhibitory and bactericidal concentration of OXY were evaluated against reference strains of *Moraxella* spp. to determine the antimicrobial concentration to use in antibacterial assays. Moreover, **ZnTMeP** and OXY alone and in association were evaluated in the formation and consolidated biofilms of all strains. The **ZnTMeP** porphyrin (4.0 μM) had no effect on formation or against consolidated biofilms. OXY (2.0 μg/mL) was efficient in reducing the production of biofilms of all *Moraxella* spp., partially removed consolidated biofilm of *M. bovoculi* and *M. ovis*, but it was unable to destroyed consolidated biofilm of *M. bovis*. The combination of porphyrin and antibiotic against both biofilms showed no synergistic effect. Then, **ZnTMeP** and OXY isolated or in combination were not efficient to completely eliminate *Moraxella* spp. in consolidated and forming biofilms. Future studies are needed using higher doses of porphyrin and/or longer exposure to light and porphyrin association with different antibiotics.

Keywords: antibiotic; **ZnTMeP**; photodynamic therapy; pinkeye; planktonic cells; sessile cells

Introduction

Infectious bovine keratoconjunctivitis (IBK) is a very important and common ocular disease of cattle. The primary agent is *Moraxella bovis* [1], but *Moraxella bovoculi* and *Moraxella ovis* can be isolated from affected animals [2, 3]. Affected animals presents lacrimation, photophobia, corneal edema and ulceration, ocular pain, and blindness [4], which causes significant economic and productive losses, expenses with repetitive treatments, commercial devaluation, and eventual discard of animals that present severe and permanent ocular sequelae [5, 6].

M. bovis, *M. bovoculi* and *M. ovis* involved in IBK cases are able to form biofilms [7, 8], which are defined as an association of microbial cells attached to surfaces, biotic or abiotic, involved in an extracellular matrix of polymeric substances, with nutrients captured for the formation of the matrix [9, 10]. Moreover, biofom biofilms allow *Moraxella* spp. persistence in ocular mucosa and nasal cavity, hampering its elimination by the immune system and antimicrobial treatment [9].

The conventional treatment for IBK consists of eliminating the bacteria from the ocular mucosa through systemic, topical or subconjunctival antibiotic administration. For parenteral route, oxytetracycline is usually the first choice for antimicrobial treatment of IBK [4, 11]. However, the therapeutic measures available to control IBK have limited success in eradicating the biofilm and the carrier state [6, 12], and repetitive antibiotic administration is required in an attempt to control the progression of the disease [4].

The biofilm leads to a large increase in resistance to antimicrobial agents compared with cultures grown in suspension, with up to 1000-fold decreases in susceptibility [7, 9]. It is investigated the possible lack of

antimicrobial penetration as an explanation of biofilm resistance and a number of delivery strategies have been used in an attempt to drive antimicrobial agents through biofilms [13].

In this case, the use of photodynamic therapy (PDT) has been evaluated as an alternative method to inactivate bacteria in biofilms [14-16]. This therapy is based on the action of a photosensitive compound, as porphyrins, which is activated by a specific wavelength of light, producing reactive oxygen species, as free radicals and/or singlet oxygen, that are able of inducing cell apoptosis or photo-oxidizing proteins, lipids and DNA, inactivating or altering several cellular molecules [17, 18].

Therefore, this study aimed to evaluate the antimicrobial activity of PDT using a zinc (II) porphyrin (**ZnTMeP)** and oxytetracycline hydrochloride (OXY) alone and in association, against biofilms of *M. bovis*, *M. bovoculi* and *M. ovis* in formation and consolidated, since these compounds are efficient in inactivate planktonic *Moraxella* spp. cells [7, 19].

Material and methods

The study was carried out with three reference strains of *Moraxella* spp. (*M. bovis*: ATCC® 10900; *M. bovoculli*: ATCC® BAA1259 and *M. ovis*: ATCC® 19575). In the first assay, minimal inhibitory (MIC) and bactericidal concentration (MBC) of OXY ($C_{22}H_{24}N_2O_9$, MW: 496.89 g/mol; Sigma®, St. Louis, MO, USA) were determined for the three reference strains of *Moraxella* spp. to identify the ideal antimicrobial concentration for the following assays. MIC was performed using the broth microdilution method in 96-well microplates, based on Clinical and Laboratory Standards Institute (CLSI, 2008) with adaptations [20]. For this, the microorganisms were cultured in 5% sheep blood agar at 37°C for 24 h, and a bacterial colony was transferred to brain heart infusion (BHI) broth (Kasvi®), then it was homogenized. From the inoculated BHI broth, a 10 µL aliquot (~$1x10^4$ CFU/ml) was transferred to each well containing different concentrations of OXY (0.031 to 65 µg/ml). MIC value was defined as the lowest concentration of OXY able to inhibit the visible development of the microorganism on the microplate, using 30 µL of 0.02% resazurin (Sigma®, St. Louis, MO, USA) as developer. MBC value was defined as the lowest concentration of OXY in which bacterial growth was not evidenced after plating the 10µl MIC solution (previously addition of resarzurin) on BHI agar incubated at 37°C for 48 h. All the tests were performed in triplicate.

In the second assay, the antibacterial activity of **ZnTMeP** porphyrin and OXY was evaluated separately on forming (plaktonic cells) and consolidated biofilms (sessile cells). To evaluate the antibacterial activity on forming biofilms (plaktonic cells), 100 µL of TSB plus 1% glucose, previously inoculated with each of the bacterium under test (~$1x10^4$ CFU per mL), and 100 µL of a solution containing **ZnTMeP** porphyrin (4.0 µM, concentration established by Seeger et al. [19]) or OXY (2.0 µg/mL) were added to each well of their respective microplates. Microplates containing **ZnTMeP** porphyrin were exposed to light for 30 min (treatment) or kept in the dark (control), according to conditions described by Seeger et al. [21]. TSB with 1% sterile glucose was used as the negative control as described by Marques et al. [22]. Then, all plates were incubated at 37°C for 48 h for the growth and adherence of bacterial cells. The non-adhered cells were removed with three successive washes with sterile distilled water (200 µL). A solution of 0.25% crystal violet (100 µL) was added to each well of the plate for 5 min and three washes were performed with sterile distilled water (200 µL). Next, the plate was dried at room temperature. The adhered bacteria were resuspended in 80:20 alcohol-acetone to perform optical density

spectrophotometer reading (OD_{550}) (Thermo Scientific, Waltham, MA, USA). To verify the ability to eradicate consolidated biofilms (sessile cells), microplates containing each *Moraxella* spp. strains were prepared (~$1x10^4$ CFU per mL) and incubated at 37°C for 48 h for the growth and the biofilm formation. After this period, microplates were washed with sterile distilled water (200 µL) for the removal of the planktonic cells. Then, 100 µL of TSB plus 1% glucose and 100 µL of a solution of each compound were added in each well/plate. Microplates containing **ZnTMeP** porphyrin were exposed to light for 30 min, and the microplate containing OXY was incubated at 37°C for 24 h. The following procedures were identical to those described for the evaluation of antibacterial activity against biofilm in formation.

In the last assay, the inactivation on forming and consolidated biofilms were assessed through the association of porphyrin and OXY, in order to verify a possible synergistic mechanism. All procedures were performed according previously described, except the antibacterial solutions added in the plates. It used a fixed concentration of **ZnTMeP** porphyrin (4.0 µM) associated or not with different OXY concentrations (0.25, 0.5, 1.0 and 2.0 µg/mL), totalizing 100 µl/well of solution.

Results and discussion

The MIC and MBC of OXY against all strains of *Moraxella* spp. was 2.0 µg/mL. This concentration was used when the antibiotic was applied alone in the experiment. In the association assays, lower doses (MIC, MIC/2, MIC/4 and MIC/8) were tested to verify if the association would be efficient in eliminating biofilms even with reduced doses of OXY. The concentration of **ZnTMeP** porphyrin used in the assays was established in the studies of Seeger et al. [19] and Ries et al. [23].

PDT using **ZnTMeP** porphyrin at 4 µM had no effect on forming (plaktonic cells) and consolidated biofilms (sessile cells) (Fig. 1 and 2), although its significant effect has already been demonstrated in planktonic cells and in an *ex vivo* model [19]. However, OXY was able to reduce the biofilm production in the three *Moraxella* spp. strains (*M. bovis, M. bovoculi* and *M. ovis*) when compared to the respective strains under the same growth conditions without the addition of the antibiotic (Fig. 1), and promotes a significant reduction in consolidate biofilm (sessile cells) of *M. bovoculi* and *M. ovis* (Fig. 2). OXY did not remove the consolidated biofilm (sessile cells) of *M. bovis* (Fig. 2). Although OXY was more efficient in preventing the formation and/or partially removing *Moraxella* spp. biofilms, it was unable to completely eliminate the biofilms. Studies show that the complete inactivation of *Moraxella* spp. biofilms require up to 1000x the concentration of OXY obtained in the MIC, which corroborates the difficulty of eliminating the microorganisms with the antibiotic routinely used in the treatment of IBK [7].

The combination of compounds did not present a significant effect on biofilm formation (planktonic cells) and/or in the reduction of biofilms formed (sessile cells) by *Moraxella* spp. (Fig. 3). The attempt to associate both compounds occurred with the intention of verifying if there would be a synergism in the action of these compounds that could potentiate the removal of biofilms. This type of association has already been tested on other occasions and positive results have been obtained [24]. The highly antibiotic resistant property of biofilms immediately demands for potent antimicrobial agents and novel strategies to fight against these microorganism communities [25].

Although significant progress has been made over the years in IBK research, currently, there are few studies evaluating possible new treatments for the disease. The treatment of IBK relies on antibiotics and sometimes, in alternatives to antibiotics with unknown efficacy [26]. The therapy of ocular infection is based in topical treatment, with several applications per day, to maintain the therapeutic levels of antimicrobial [11]. Also, the intramuscular administration of antimicrobials, such as OXY, is an alternative. In our study, the OXY showed positive results *in vitro* in the inactivation of planktonic cells of reference strains of *Moraxella* spp. Earlier studies have demonstrated the incidence of keratoconjunctivitis and a decreased duration of bacterial shedding when OXY were used as intramuscular treatment [27]. However, the inactivation of consolidated biofilms remains a challenge for the treatment of several diseases. In our research, OXY was unable to destroy consolidated biofilms of *M. bovis,* but showed more promising results for biofilms of *M. ovis* and *M. bovoculi* than PDT using **ZnTMeP** porphyrin. Our results corroborate previous studies that demonstrate the difficulty of inactivating biofilms formed by *Moraxella* spp. [7, 8].

Biofilms have several advantages for the microorganism survival compared with planktonic cells, such as a higher virulence, physical and chemical heterogeneity, and as consequence an increased tolerance towards the conventional treatments [28]. In agreement with this statement, Prieto et al. [7] demonstrated that the concentration of the antibiotic needed to effectively inhibit the growth reach over 1000-fold higher than the figure for bacteria in suspension, therefore the MIC values fail to reflect the bacterial sensitivity to antibiotics in biofilms.

Several crucial elements in the infectious process and formation of biofilm have been proposed for application of novel technologies and drug delivery systems, aiming the prevention of colonization and biofilm formation [29]. Then, some studies have demonstrated the potential of using PDT combined with antibiotics against bacteria to increase the efficiency of bacterial inactivation [24]. The PDT could be considered a safer treatment to the host. This method takes advantage of a proper combination of a local treatment, an effective (intra) extracellular antioxidant system in the healthy mammalian cells and the photosensitizer intracellular location [30].

According to our antibacterial assays, the **ZnTMeP** porphyrin did not demonstrate to be able to prevent the formation of biofilms nor to inactivate them once they are already established. These results agree with previous studies that claim that biofilms a lower susceptibility against PDT comparatively to their equivalent planktonic forms, and therefore, to successfully photoinactivate these complex structures could be necessary higher concentrations of photosensitizer and/or longer time of treatment are required [28]. Unfortunately, **ZnTMeP** porphyrin had no action on biofilms, different from what was expected, since PDT using this porphyrin had an excellent action in inactivating planktonic bacteria of *Moraxella* spp. after 2.5 minutes of exposure to light [19].

Despite the synergistic combination of antibiotics with PDT was reported in some microorganisms as *Staphylococcus aureus* and *Helicobacter pylori* [31, 32], in our assay the **ZnTMeP** porphyrin showed no additive effect on inactivating developing and consolidated biofilms, even when the antibiotics were used. The main target of PDT is the external structures of the bacteria, such as cell membrane and cell wall, so the porphyrins can act allowing an easier enter of the antibiotics into the bacterial cells [24]. Thus, an antibiotic with action on the cell wall of bacteria could be a more efficient option in presenting a synergistic effect.

Conclusion

In summary, we demonstrated that **ZnTMeP** porphyrin did not show satisfactory results in inactivating biofilms of *Moraxella* spp. from cattle, even associated with antibiotics. However, OXY alone presented partial activity against *Moraxella* spp. biofilms, but was unable to destroy the consolidated biofilm (sessile cells) of *M. bovis*. Then, future studies with higher doses of porphyrin or with longer exposure to light are necessary and new tests with antibiotics that interfere with bacterial cell wall synthesis are suggested.

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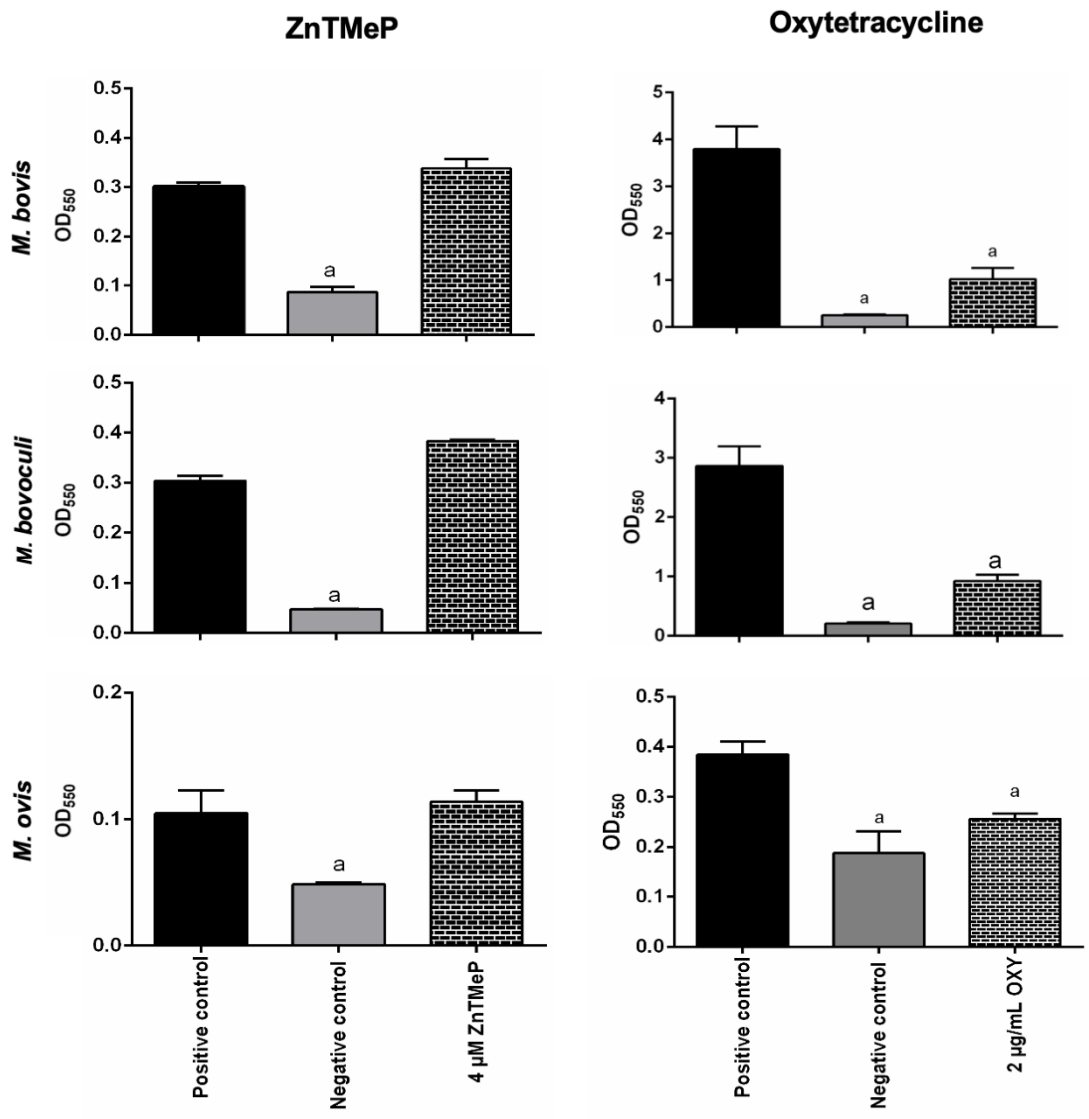

Fig. 1: ZnTMeP porphyrin and oxytetracycline individual activity against biofilms in formation (planktonic cells) of *Moraxella* spp. from cattle. a: indicates statistical difference when compared with positive control (p<0.05).

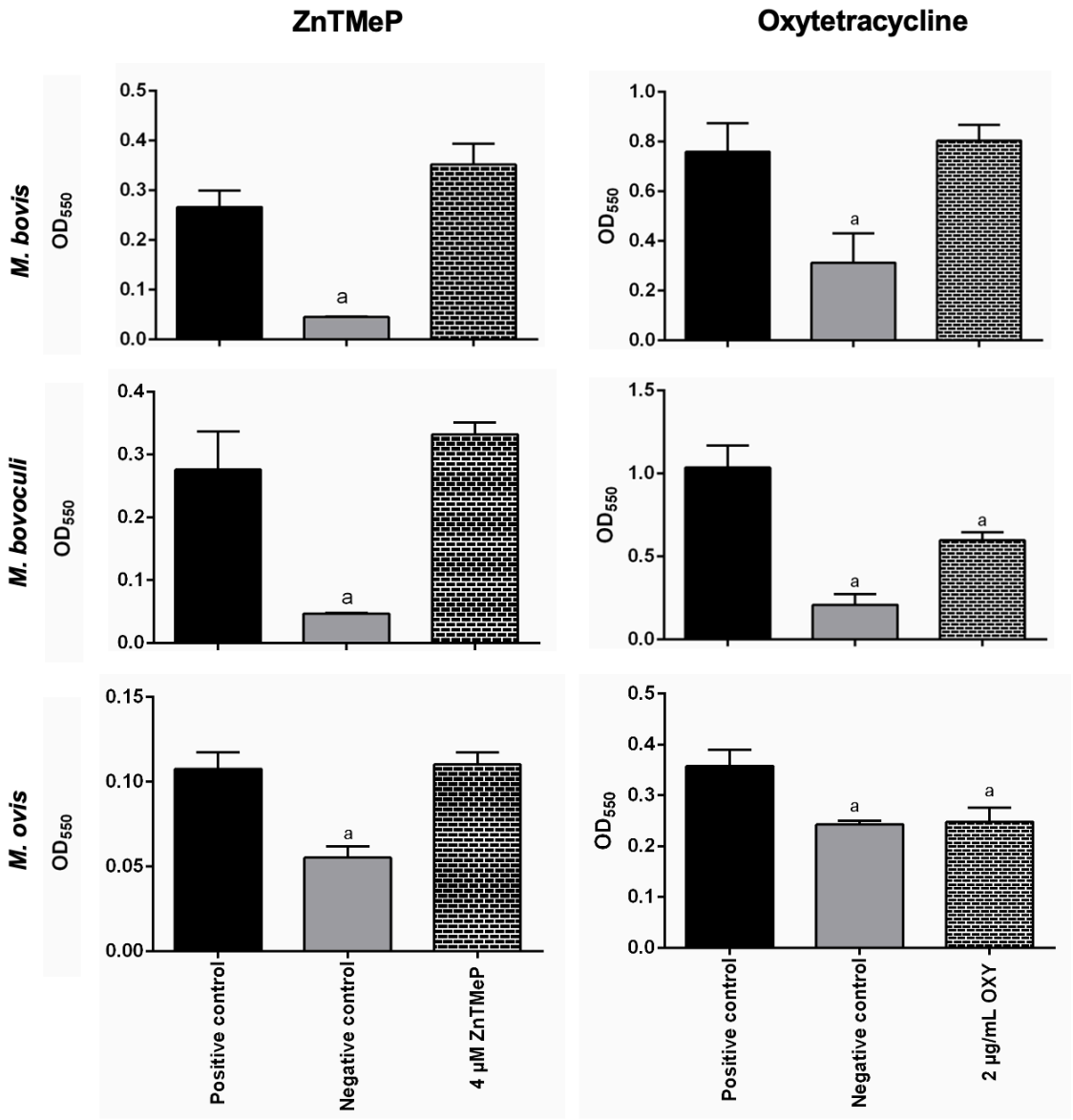

Fig. 2: ZnTMeP porphyrin and oxytetracycline individual activity against consolidated (sessile cells) biofilms of *Moraxella* spp. from cattle. a: indicates statistical difference when compared with positive control (p<0.05).

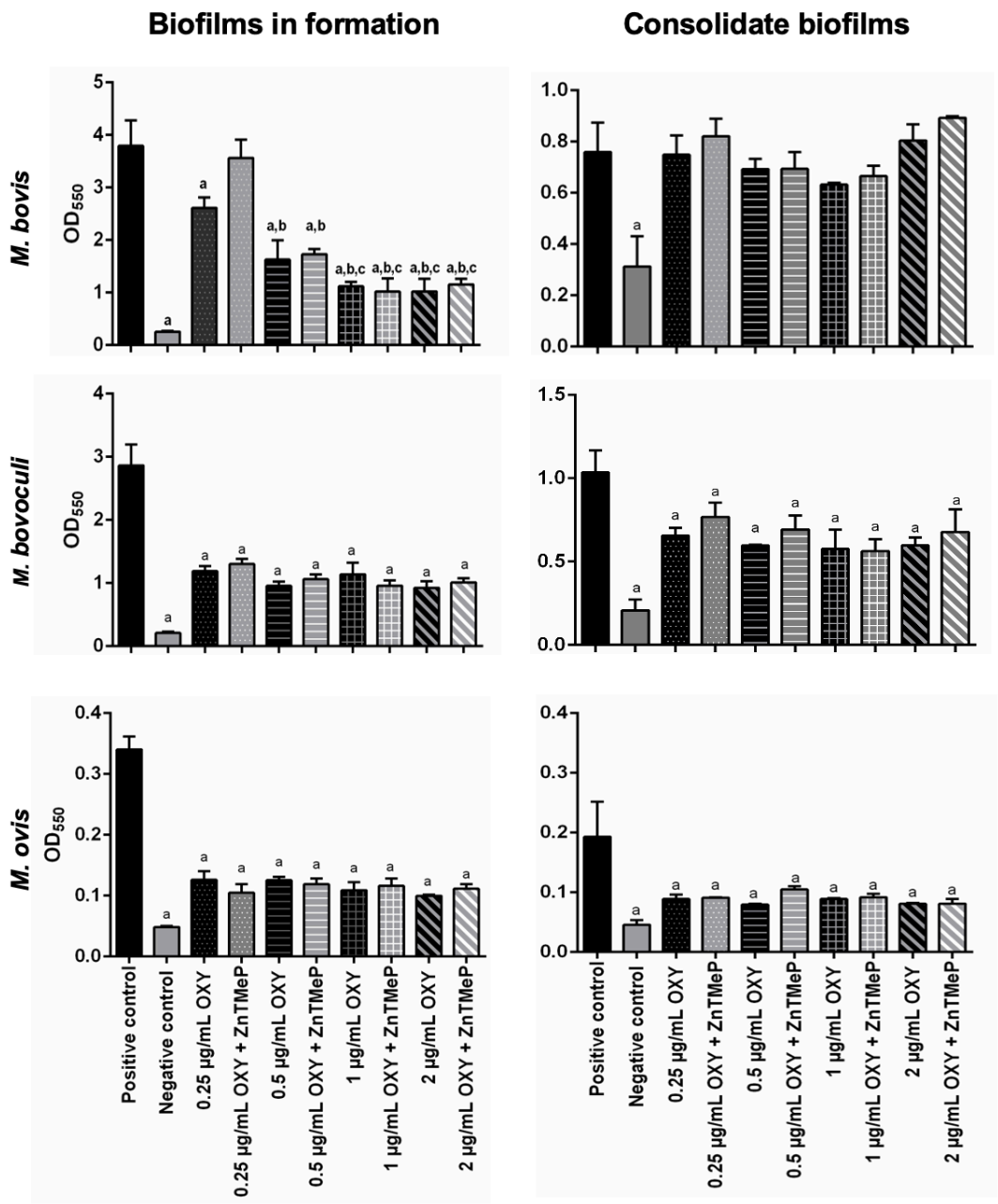

Fig. 3: Antibacterial activity of **ZnTMeP (**4.0 µM) and oxytetracycline hydrochloride isolated and in combination against biofilms in formation (planktonic cells) and consolidate (sessile cells) of *Moraxella* spp. from cattle. a: indicates statistical difference from the positive control (p<0.05); b: statistical difference from 0.25 µg/mL OXY (p<0.05); c: statistical difference from 0.5 OXY and 0.5 OXY + **ZnTMeP** (p<0.05).

4 CONSIDERAÇÕES FINAIS

A ceratoconjuntivite infecciosa bovina é a doença ocular mais comumente encontrada em animais de produção, que resulta em diferentes graus de lesão ocular e, apesar de não ser uma doença fatal, causa prejuízos econômicos significativos na produção de bovinos. O tratamento para essa enfermidade apresenta diversas dificuldades, como a falta de fármacos que apresentem resultados rápidos e que eliminem o agente do rebanho, tornando recorrentes os casos de reaparecimento da doença. Assim, medidas alternativas para o tratamento da doença são necessárias.

A terapia fotodinâmica antimicrobiana vem sendo reconhecida como uma alternativa muito promissora ao tratamento com antimicrobianos, especialmente em infecções localizadas. Esse método, tem como base a ação de um composto fotossensível (como porfirinas, azul de metileno, verde malaquita, etc.) que, quando ativado por diferentes comprimentos de onda de luz, produz espécies reativas de oxigênio que são capazes de induzir a apoptose e/ou morte celular.

Assim, nessa dissertação foi avaliada a atividade *in vitro* de duas porfirinas tetracatiônicas frente a isolados e cepas padrão de *Moraxella* spp. Os resultados do ensaio *in vitro* encorajaram a avaliação da atividade de uma das porfirinas (**ZnTMeP**) em modelo *ex vivo* de globo ocular de bovinos. Tantos os resultados *in vitro* como em modelo *ex vivo* foram promissores e, por isso, foi investigada a ação da terapia fotodinâmica usando a porfirina **ZnTMeP** sobre biofilmes de *Moraxella* spp., uma vez que essas bactérias tem capacidade de formar biofilmes e permanecer na conjuntiva e no canal lacrimal de bovinos infectados, o que dificulta o tratamento e o controle da enfermidade.

Dessa forma, os resultados obtidos nessa dissertação permitem concluir que:

- *In vitro,* a porfirina **ZnTMeP** foi efetiva em inativar completamente os isolados clínicos e cepas padrão de *Moraxella* spp. em até 7,5 min de irradiação, sugerindo que esse composto possa ser eficiente em eliminar células planctônicas de *Moraxella bovis*, *M. ovis* e *M. bovoculi* em animais;

- A porfirina **H_2TMeP** não apresentou atividade antibacteriana *in vitro* contra os isolados de *Moraxella* spp., mesmo após 7,5 minutos de exposição à luz;

- No modelo *ex vivo* de globo ocular de bovinos, a porfirina **ZnTMeP** promoveu uma redução significativa na concentração bacteriana de todas as cepas de *Moraxella* spp. testadas, após 30 min de irradiação. A inativação bacteriana não foi completa e o tempo de inativação

em modelo *ex vivo* foi superior ao observado no ensaio *in vitro*, mas ainda assim os resultados foram significativos e indicaram eficiência da terapia fotodinâmica frente a *Moraxella* spp.

- A porfirina **ZnTMeP** não teve efeito antibacteriano na formação ou na redução de biofilmes consolidados de *M. bovis*, *M. ovis* e *M. bovoculi*, indicando que, na concentração avaliada, a ação dessa porfirina é restrita às células planctônicas. Assim, é fundamental que continuem sendo investigadas outras alternativas que possam ser eficientes sobre as comunidades bacterianas dispostas em biofilmes (novas moléculas, associação de terapias, etc.)

- A oxitetraciclina (OXY) foi avaliada individualmente e associada à porfirina **ZnTMeP** com o intuito de pesquisar o efeito sinérgico dos compostos sobre biofilmes de *Moraxella* spp., uma vez que a terapia fotodinâmica isolada não foi eficiente. A OXY foi eficiente na redução da produção de biofilmes de todas as cepas de *Moraxella* spp. avaliadas no estudo, e removeu parcialmente o biofilme consolidado de *M. bovoculi* e *M. ovis,* mas não foi capaz de destruí-los completamente.

- Assim como a porfirina **ZnTMeP**, a OXY não foi eficiente em destruir biofilmes consolidados de *M. bovis*, confirmando que a eliminação de biofilmes ainda é um desafio e que necessita de medidas mais eficientes do que os utilizados rotineiramente no tratamento dos animais;

- A combinação de **ZnTMeP** e OXY não apresentou efeito sinérgico na atividade frente a biofilmes em formação e em biofilmes consolidados de *Moraxella* spp.

As limitações que podem existir na utilização da terapia fotodinâmica com porfirinas, incluem o tempo de exposição que é necessário para ocorrer a ativação dos compostos e seu efeito na inativação dos micro-organismos, necessitando de mais estudos para a padronização de um protocolo ideal e a presença de luz que é essencial para a ativação dos compostos.

De forma geral, pode-se concluir que a porfirina **ZnTMeP** tornou-se uma opção interessante para o desenvolvimento de futuras pesquisas *in vivo* utilizando a terapia fotodinâmica em bovinos com ceratoconjuntivite infecciosa. No entanto, ainda é urgente a busca por novos compostos e terapias que possam ser efetivas em interferir na formação e na destruição de biofilmes de *Moraxella* spp.

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