UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

AÇÃO ANTIMICROBIANA DE PRODUTOS NATURAIS E SINTÉTICOS E EFEITO SINÉRGICO COM PENICILINA, *IN VITRO*, CONTRA C*orynebacterium pseudotuberculosis* BIOVAR *ovis*

PEDRO NEGRI BERNARDINO

Botucatu, SP
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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus Botucatu como requisito para obtenção do grau de Mestre.

Orientador: Prof. Dr. José Paes de Oliveira Filho.

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ddNTP – dideoxinucleotídeo

CIM – Contração Inibitória Mínima

CBM – Concentração Bactericida Mínima

LAC – Linfadenite Caseosa

MIC – *Minimum Inhibitory Concentration*

MBC – *Minimum Bactericidal Concentration*

ddATP – 2’3’-dideoxiadenosina

ddGTP – 2’3’-dideoxiguanina

ddCTP – 2’3’-dideoxicitosina

ddTTP – 2’3’-dideoxitimina

SUMÁRIO

Bernardino, P.N. **Ação antimicrobiana de produtos naturais e sintéticos e efeito sinérgico com penicilina, *in vitro*, contra C*orynebacterium pseudotuberculosis* biovar *ovis*.** Botucatu, 2019, 55p., dissertação de mestrado, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), campus de Botucatu.

RESUMO

Infecções por *Corynebacterium pseudotuberculosis* são comuns em pequenos ruminantes e representam risco ocupacional para humanos. Pelas opções de tratamentos serem escassas e apresentarem alto índice de recidiva ou baixa eficácia, a busca por novas alternativas se torna importante. Alguns compostos sintéticos - como os dideoxinucleotídeos (ddNTPs) - e naturais - como própolis, cinamaldeído, eugenol, carvacrol e timol - possuem efeito antimicrobiano comprovado para diferentes bactérias e fungos, porém não existem estudos em isolados de *C. pseudotuberculosis*. Foram realizados testes *in vitro* por cultivo em diluição seriada de cada um dos componentes supracitados para determinação da Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM), além de utilizá-los em conjunto com a penicilina para avaliação de possível sinergismo. A 2'3'-dideoxiadenosina, 2'3'-dideoxiguanina, própolis e cinamaldeído apresentaram ação antimicrobiana quando utilizados separadamente e também efeito sinérgico em conjunto com a penicilina. Estes resultados indicam potencial de utilização destes quatro produtos como alternativas de tratamento de *C. pseudotuberculosis*.

Palavras-chave: dideoxinucleotídeos, própolis, cinamaldeido, penicilina, sinergismo.

Bernardino, P.N. **Antimicrobial action of natural and synthetic products and synergic effect with penicillin, *in* vitro, against *Corynebacterium pseudotuberculosis* biovar *ovis*.** Botucatu, 2019, 55p., master's degree dissertation, School of Veterinary Medicine and Animal Science (FMVZ), Sao Paulo State University (UNESP), Botucatu campus.

ABSTRACT

Corynebacterium pseudotuberculosis infections are common in small ruminants and present as an occupational hazard for humans. Treatment options are scarce and present high chances of recurrence or low efficacy, making the demand for new alternatives important. Some synthetic compounds, like dideoxynucleotides (ddNTPs), and natural compounds, such as propolis, cinnamaldehyde, eugenol, thymol and carvacrol, have verified antimicrobial effect for multiple strains of bacteria and fungi, but not against *C. pseudotuberculosis*. *In vitro* tests by culture in serial dilution of each compound mentioned were performed to determine a Minimum Inhibitory Concentration and Minimum Bactericidal Concentration (MIC and MBC), besides tests using them in association with penicillin for evaluation of possible synergism. 2'3'-dideoxyadenosine, 2'3'-dideoxyguanine, propolis and cinnamaldehyde presented antimicrobial action when used separately as well as synergic effect associated with penicillin. These results indicate a potential for therapeutic use of the four products as treatment options for *C. pseudotuberculosis*.

Key-words: dideoxynucleotides, propolis, cinnamaldehyde, penicillin, synergism.

CAPÍTULO 1

1. Introdução

Corynebacterium pseudotuberculosis é agente gram positivo, intracelular facultativo pleomórfico, com formato cocoide ou de bacilos filamentosos, desprovido de flagelos, não encapsulado, não esporulado e anaeróbio facultativo (DORELLA et al., 2006). É capaz de sobreviver no ambiente por períodos entre oito até 55 dias quando incorporados em alimentos, como feno, ou mesmo em fezes e lascas de madeira (AUGUSTINE & RENSHAW, 1986; SPIER et al., 2012). São organismos sensíveis a grande parte dos desinfetantes e antissépticos, como amônia quaternária, clorexidine, iodo e cloro (SÁ et al., 2013).

O longo período de sobrevivência de *C. pseudotuberculosis* no ambiente favorece a disseminação do patógeno, facilitando a infecção de animais e humanos, causando a Linfadenite Caseosa (LAC). A transmissão ocorre pela contaminação de feridas advindas de manejo, procedimentos veterinários ou contato entre animais do mesmo rebanho, possuindo outras vias de infecção, como ingestão ou aspiração da bactéria excretada em material caseoso ou até mesmo pela tosse de indivíduos infectados (WILLIAMSON, 2001). Em humanos, apresenta um risco ocupacional decorrente da exposição do indivíduo à bactéria no ambiente laboral, como fazendas, abatedouros ou laboratórios (PEEL et al., 1997; HEGGELUND et al., 2015).

A superfície lipídica com ácido micólico e a produção da exotoxina fosfolipase D são os principais fatores de virulência que determinam a patogenia de *C. pseudotuberculosis*, sendo a primeira responsável pela proteção contra ação dos fagócitos e a segunda pela indução do potencial de invasão ao organismo por meio do aumento da permeabilidade capilar, inflamação e necrose (BAIRD & FONTAINE, 2007). A sintomatologia consiste na inflamação de linfonodos regionais e formação de abscessos com conteúdo caseoso, que podem supurar, no subcutâneo ou nos linfonodos, tanto em animais quanto em humanos (DORELLA et al., 2006; ZAVOSHTI et al., 2012; BASTOS et al., 2012).

Técnicas diagnósticas para a LAC consistem no cultivo e isolamento da bactéria a partir de conteúdo de abscessos, inoculação em animais de laboratório, detecção da bactéria a partir de métodos biomoleculares (como a PCR) e testes sorológicos dos indivíduos com suspeita de infecção (OREIBY, 2015).

Vacinas atenuadas e inativadas, além das compostas por toxoides ou DNA, ou a combinação de toxoides com a bactéria inativada, encontram-se disponíveis no mercado.

Não há imunização completa dos rebanhos vacinados, entretanto, observa-se manifestação mais branda da doença e redução da incidência da mesma (WINDSOR, 2011).

2. Revisão de Literatura

O tratamento sistêmico da LAC caprina e ovina com antimicrobianos não apresenta boa resposta devido à proteção que os abscessos formados conferem às bactérias que ficam em seu interior. Porém, o uso prolongado de antimicrobianos (4 a 6 semanas) tem sido um método de tratamento convencional da LAC (WILLIAMSON, 2001; GUIMARÃES et al., 2011). *C. pseudotuberculosis* apresenta alta sensibilidade *in vitro* a maioria dos antimicrobianos disponíveis para uso veterinário, como penicilina, ceftiofur e tetraciclina. No entanto, quando forma biofilmes, que hipoteticamente mimetizariam a ação da barreira de um abscesso, a sensibilidade diminui consideravelmente - mais que 500 vezes - a concentração inibitória mínima (OLSON et al., 2002). Alternativas terapêuticas já foram estudadas, como o uso da terapia antimicrobiana fotodinâmica, que consiste na aplicação de agentes fotodinâmicos, que ao receberem estímulos luminosos, induzem a produção de espécies reativas de oxigênio com ação localizada, resultando na cura dos indivíduos sem recidivas (SELLERA et al., 2016). A remoção cirúrgica dos abscessos formados é uma opção, além da combinação com o uso de antimicrobianos, principalmente em animais com alto valor genético, apesar das chances de recidiva serem altas a longo prazo (GEZON et al., 1991).

Para controle eficaz no rebanho, atualmente é recomendado o diagnóstico clínico e sorológico de indivíduos infectados, com posterior remoção dos mesmos do rebanho (BAIRD & MANOLE, 2010; JESSE et al., 2015). A vacinação, apesar de não apresentar elevada eficácia, demonstra potencial de controle da doença, podendo ser opção para melhor profilaxia dentro de uma propriedade, com consequente diminuição de perdas econômicas (WINDSOR & BUSH, 2016).

Encontrar métodos de tratamento para a LAC se torna importante por visar a preservação da saúde humana, pelo potencial zoonótico da bactéria, e a manutenção de animais no rebanho sem apresentarem recidivas, principalmente daqueles com alto valor genético.

2.1. Dideoxinucleotídeos (ddNTPs)

Os ddNTPs são nucleotídeos com mudanças estruturais capazes de inibir a açãoo de enzimas celulares, apresentando potencial terapêutico (JORDHEIM et. al., 2013). Estas mudanças são observadas no anel de pentose do nucleosídeo (TSESMETZIS et al., 2018), mais especificamente pela substituição do grupo hidroxila por um hidrogênio na porção 3’ do anel. Essas moléculas sintéticas atuam como terminadores de cadeia durante a replicação de DNA, bloqueando o correto funcionamento da DNA-polimerase por não possibilitarem a ligação de nucleotídeos subsequentes (TOJI & COHEN, 1969).

Testes com a 2’,3’-dideoxiadenosina revelaram ação antibacteriana *in vitro* em várias enterobactérias e outras espécies comumente isoladas em cultivos microbiológicos, avançando para ensaios laboratoriais que sugeriram boa ação *in vivo* em roedores (DOERING et al., 1966; BESKID et al., 1981). No entanto, não há estudos com estes compostos em isolados de *C. pseudotuberculosis*.

Apesar dos resultados obtidos para terapia antibacteriana, os ddNTPs e outros análogos de nucleotídeos passaram a ser utilizados em outros campos da ciência, como para o sequenciamento de material genético (SANGER et al., 1977) ou para terapias antivirais, antineoplásicas e imunossupressoras (FUNG et al., 2002; GALMARINI et al., 2002; COLOMBEL et al., 2010). Entretanto, com o surgimento de agentes resistentes à antibioticoterapia convencional, estudos dos análogos de nucleotídeos, como a pseudoridimicina, demonstram resultados positivos quando testados contra bactérias (MAFFIOLI et al., 2017; CHELLAT & RIEDL, 2017), ressaltando a importância do conhecimento dos possíveis efeito de tais substâncias, como os ddNTPs, e uso potencial no tratamento de bactérias.

2.2. Compostos naturais

Dentre os produtos naturais atualmente estudados pela ação antimicrobiana, encontramos óleos essenciais, polipeptídeos produzidos por microrganismos e substâncias apícolas complexas.

2.2.1. Própolis

A própolis é um produto sintetizado pelas abelhas que utilizam secreções de árvores, folhas, flores e pólen, resultando em composição complexa de cera, resina, bálsamos, óleos voláteis e impurezas (BANKOVA et al., 2000). Tal composto já demonstrou atividades terapêuticas, incluindo ações antimicrobiana, imunomoduladora, antinflamatória e antitumoral (SFORCIN et al., 2000; SFORCIN et al., 2001; FREITAS et al., 2006; BÚFALO et al., 2009; ORSATTI et al., 2010; WATANABE et al., 2011).

RAHMAN et al. (2010) investigaram a ação da própolis em *Staphylococcus aureus* e *Escherichia coli*, indicando a concentração inibitória mínima (MIC) e concentração bactericida mínima (MBC) de 2,74 e 5,48 mg/mL, respectivamente. Já extrato de própolis advindo de Omã resultou em uma MIC menor que 0,1 mg/mL para *S. aureus* e menor que 0,38 mg/mL para *E. coli* (POPOVA et al., 2013). Para isolados dentais de grupos estreptococócicos mutantes e *Lactobacillus* spp., a própolis polonesa apresentou MIC de 1,10 e 0,7 mg/mL e MBC de 9,01 e 5,91 mg/mL, respectivamente (DZIEDZIC et al., 2013).

Em isolados de *C. pseudotuberculosis*, assim como outras bactérias, gram positivas e negativas, foi testada a ação do mel de diferentes regiões da Arábia Saudita, que apresentaram atividade bactericida parecida com a tetraciclina para todas as espécies incluídas no estudo, provavelmente devido a presença de própolis no mel (HEGAZI & ALLAH, 2012). Outros grupos de corinebactérias também foram testados: entre agentes causadores de mastite, *C. bovis* foi isolado e apresentou resistência contra diversos antibióticos, como a penicilina, e também demonstrou ser resistente à própolis (HEGAZI et al., 2014); *C. striatum* apresentou MIC entre 63 e 90 µg/mL para uma própolis francesa (BOISARD et al., 2015); já a MIC para *C. diphtheriae* foi de 50 µg/mL de própolis da Turquia (BAYRAM et al., 2015).

2.2.2. Cinamaldeído

Composto aldeído aromático, o cinamaldeído é encontrado na casca da canela e apresenta ações antibacteriana e antifúngica. Ooi et al. (2006) descreveram valores de MIC entre 75 e 650 µg/mL para alguns fungos filamentosos, leveduras e bactérias. Tais valores foram diferentes entre os isolados ao atingir MIC de 0,78 a 50 µg/mL (SANLA-EAD et al., 2012). Não obstante, estudos apresentaram resultados de 2 µg/mL como valor da MIC, além de não haver desenvolvimento de resistência bacteriana com a passagem de culturas de *Helicobacter pylori (*ALI et al., 2005).

Além da atuação como antimicrobiano, o cinamaldeído apresentou propriedades hipoglicemiantes e hipolipidêmicas, possibilitando o seu uso como agente antidiabético (BABU et al., 2007). A possível ação imunomoduladora também pôde ser observada pela inibição de algumas citocinas de macrófagos e monócitos de roedores, conferindo caráter antioxidante e anti-inflamatórios (CHAO et al., 2008).

Na literatura consultada, não foram encontrados estudos considerando a ação antibacteriana desta substância em *C. pseudotuberculosis*.

2.2.3. Eugenol

Composto presente no óleo essencial do cravo (*Eugenia caryophillis*), também demonstrou apresentar ação antimicrobiana. Contra *Helicobacter pylori*, inibiu o crescimento em 9 horas de exposição na MIC de 2 µg/mL, não desenvolvendo resistência bacteriana após sucessivos cultivos, assim como o cinamaldeído (ALI et al., 2005). Através do rompimento da membrana plasmática da *Salmonella typhi*, o eugenol manifestou ação antibacteriana com MIC de 12,5 µg/mL e MBC de 25 µg/mL (DEVI et al., 2010). Um possível efeito sinérgico do eugenol com antibióticos - tanto hidrofílicos quanto lipofílicos – foi testado em bactérias gram-negativas, resultando em diminuição de 5 a 1.000 vezes a MIC inicial dos antibióticos avaliados, possivelmente pela ação na membrana plasmática bacteriana (HEMAISWARYA & DOBLE, 2009). O efeito antifúngico foi revelado em isolados de onicomicoses (GAYOSO et al., 2005), fungos de madeira (CHENG et al., 2008) e *Candida albicans* (HE et al., 2007).

Em estudos parasitológicos, observou-se ação anti-helmíntica ao inibir a eclosão de ovos de *Haemonchus contortus* (PESSOA et al., 2002). Ainda, o eugenol agiu como potencial agente contra *Leishmania amazonenses*, com MIC de 150 µg/mL e, quando usado como tratamento de macrófagos de ratos antes da exposição ao protozoário a 100 e 150 µg/mL, não apresentou efeito citotóxico, além de diminuir o índice de associação macrófago com a fase promastigota, indicando-o como potencial substância terapêutica contra a leishmania (UEDA-NAKAMURA et al., 2006).

Além de antimicrobiano, já foram relatadas ações antinflamatória, anticonvulsivante, vasodilatadora, antioxidante, anestésica, analgésica e antineoplásica (PRAMOD et al., 2010).

Quando exposto ao *Corynebacterium* spp., o eugenol é biotransformado em metabólitos com potencial uso pelas indústrias farmacêuticas e de alimentos, como a vanilina, ácido vanílico, ácido ferúlico e ácido protocatecuico (TADASA & KAYAHARA, 1983; MISHRA et al., 2013). Informações quanto ao efeito antimicrobiano deste composto contra *Corynebacterium* não foram encontradas na literatura consultada.

2.2.4. Timol e Carvacrol

Componentes do óleo essencial do orégano, alguns efeitos biológicos do timol e do carvacrol já foram descritos. Tais componentes têm a capacidade de danificar a membrana plasmática de bactérias, como *Pseudomonas aeruginosa* e *Staphylococcus aureus*, afetando o equilíbrio iônico e ácido-base celular, apresentando ação antibacteriana (LAMBERT et al., 2001). A habilidade de aumentar a permeabilidade e desestabilizar a polarização da membrana celular também foi observada com testes realizados em *E. coli*, atingindo MIC de 200 µg/mL (XU et al., 2008). Adicionalmente, esses compostos apresentaram efeito bactericida em *S. aureus* e *S. epidermidis* e, também, eficácia na inibição e destruição de biofilmes pelas mesmas bactérias quando usados em concentrações maiores (NOSTRO et al., 2007).

Quando testado o óleo essencial do orégano ou apenas um destes dois constituintes, separadamente, foram observadas propriedades antifúngicas, herbicidas e inseticidas, algumas delas mais potentes que compostos sintéticos comercializados (KORDALI et al., 2008). Ademais, ação inibidora da acetilcolinesterase, conferindo potencial terapêutico, também foi relatada (JUKIC et al., 2007). Ainda foram detectadas possíveis atuações antineoplásicas e antioxidantes de derivados destas duas substâncias (MASTELIC et al., 2008).

Corynebacterium spp. apresentou sensibilidade ao timol quando incorporado a esferas de acetato de celulose para liberação lenta, porém apenas nas concentrações mais altas (MILOVANOVIC et al., 2016). Quando foram testados óleos essenciais com considerável fração de timol e carvacrol na composição, efeitos antibacterianos contra várias corinebactérias, entre outras bactérias, foram verificados (BEZBRADICA et al., 2005; NZEAKO et al., 2006; KUMAR et al., 2011).

CAPÍTULO 2

1. Trabalho científico 1

Trabalho a ser submetido para a revista Microbial Pathogenesis (ISSN: 0882-4010, Elsevier). Site da revista: https://www.journals.elsevier.com/microbial-pathogenesis. As normas para submissão de manuscrito podem ser encontradas no link: https://www.elsevier.com/journals/microbial-pathogenesis/0882-4010/guide-for-authors.

Antibacterial action of synthetic nucleotide analogues against *Corynebacterium pseudotuberculosis* and synergistic effect with penicillin

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Highlights

- Growth delay of *C. pseudotuberculosis* incubated with dideoxynucleotides;
- Antimicrobial action of dideoxyadenosine against *C. pseudotuberculosis*;
- Synergism of adenosine and guanine analogues with penicillin.

Abstract

Caseous lymphadenitis is a serious disease for small ruminant production and can represent a public health concern as well. In both cases, few treatment options are available and show low efficiency. The use of new products becomes of great interest for therapy of *Corynebacterium pseudotuberculosis*-induced infections disease. Dideoxynucleotides (ddNTP) already presented antibacterial action for various species, but never described for *C. pseudotuberculosis*. The present study investigated Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for the four DNA nucleotide analogues in an ATCC and a clinical isolate and evaluate a possible synergism with penicillin, an antibiotic commonly used for this type of infection. 2’3’-dideoxyadenosine (ddATP) and 2’3’-dideoxyguanine (ddGTP) showed a growth delay effect in concentrations from 0.5 to 2 µmol/mL and the first one showed MIC and MBC of 4 µmol/mL for ATCC 19410. Associated with penicillin, an antimicrobial effect was observed when using concentrations lower than the MIC of these substances individually for both strains tested. This indicates an alternative for treatment of *C. pseudotuberculosis* in animals using nucleotide analogues or the combination of some ddNTPs and penicillin, a possible therapy for other bacteria.

Keywords: *C. pseudotuberculosis*, 2’3’-dideoxyadenosine, 2’3’-dideoxyguanine, synergism, antimicrobial.

1. Introduction

Corynebacterium pseudotuberculosis is a well-known gram positive bacteria and a facultative intracellular pleomorphic organism able to infect animal and humans [1]. It is the causal agent of caseous lymphadenitis in small ruminants. The virulence factors are the lipid surface containing mycolic acid, protecting the agent against macrophages, and production of exotoxin phospholipase D, which induces higher vascular permeability, inflammation and necrosis [2]. The usual clinical signs are regional lymphnodes swelling and abscess formation in the subcutaneous region or in the lymphnodes [3,4]. Diagnostics consist in culture of caseous content of abscesses, inoculation in laboratory animals, molecular methods for bacterial DNA detection and serologic tests [5]. There are commercial vaccines available for disease control, but with moderate efficiency [6], making the early removal of clinically and/or serologically positive animals from the farms the best control method, nowadays [7,8].

Routine treatment options are surgical removal of abscesses, antibiotics therapy of long duration (4 to 6 weeks) or even alternative techniques, such as photodynamic antimicrobial therapy, presenting low efficacy or high chances of abscesses relapse [9,10,11,12,13]. This brings the need of new therapeutic methods for caseous lymphadenitis, aiming lower economic and genetic losses in sheep and goat flocks and new options for management of the agent regarding public health problems.

Dideoxynucleotides (ddNTPs) are similar to the normal deoxynucleotides but have the hydroxyl in the 3’ carbon of the pentose ring replaced by a single hydrogen molecule, turning them into chain terminators during the synthesis of DNA by blocking the link of the next nucleotide into the sequence [14,15]. Antibacterial action of the 2’,3’-dideoxyadenosine (ddATP) was already observed *in vitro* and *in vivo* [16,17] but not including corynebacteria species.

Our research focus on detecting the therapeutic potential of ddNTPs against *in vitro* cultures of *C. pseudotuberculosis* and to investigate a possible synergic action between these compounds and penicillin, considering those as possible treatment options for this infection.

2. Material and Methods

Institutional Animal Care and Use Committee approval

The study was in accordance with the policies of the Institutional Animal Care and Use Comitte (CEUA 0118/2019) of São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, Botucatu/SP, Brazil.

C. pseudotuberculosis isolates

A well-characterized and sequenced strain (ATCC 19410) was received from the Institute of Biological Sciences of Federal University of Minas Gerais and another strain was isolated from a goat abscess at the Microbiology Laboratory of the School of Veterinary Medicine and Animal Science from UNESP, Botucatu. They were stocked in a glycerinated brain and heart infusion (BHI) broth at -20°C.

ddNTPs and penicillin

2'3'-dideoxyadenosine (ddATP), 2'3'-dideoxyguanine (ddGTP), 2'3'-dideoxycitosine (ddCTP) and 2'3'-dideoxytimine (ddTTP) were purchased from Sigma-Aldrich (Cotia, SP) and the penicillin used was Aricilina® (Blau Farmacêutica S.A.; Cotia, SP).

Minimum inhibitory concentration (MIC) of ddNTPs for ATCC strain

Pilot experiments were conducted with ddATP, ddGTP, ddCTP and ddTTP diluted into a 4 µmol/mL solution and serial dilution to 50% of the concentration was made from 2 to 0.001 µmol/mL in a 96 well plate by using Muller Hinton (MH) broth with 0.2% of Tween 80 in a volume of 75 µL. Considering previous descriptions of antibacterial action and MIC values for other bacterial species provided by Beskid at al. (1981), only ddATP was used in higher concentrations (8 and 4 µmol/mL) to reach its MIC for *C. pseudotuberculosis*.

The *C. pseudotuberculosis* ATCC colonies were incubated in BHI agar and dissolved into 8.5% saline solution until turbidity reach the 0.5 McFarland standard and then added to the MH broth with 0.2% Tween 80 for a bacterial concentration of 1.5 x 10^6 bacteria/mL. 75 µL of this final solution was added to each well with the compounds in it, reaching a final volume of 150 µL/well. Positive and negative controls were made with the same amount of bacteria, for the positive, or no bacteria, for the negative, and final volume as the samples. The plate was incubated at 37°C on a shaking platform (220 rpm) in aerobic conditions during 48 hours. Three evaluations were made: first, a subjective turbidity evaluation of each well; second, an optic density reading by a spectrophotometer (Epoch™ 2, BioTech®), on a 600 nm wave length; and third, a resazurin reduction test.

50 µL of 0.05% resazurin was added into each well, incubated for 2 hours at the same previous conditions and observed the color of the final solution where pink indicates the presence of bacterial growth and blue indicates growth inhibition. The comparison of the three results gave the MIC of the ddNTPs.

Minimum bactericidal concentration (MBC) of ddNTPs for ATCC strain

Before the resazurin test, the wells with no apparent growth by presenting low optic density and clear solution were cultivated in a BHI agar petri dish using calibrated handles, as well as the positive control, at 37°C in aerobic conditions during 48 hours and then evaluated according to the colony growth. The MBC was considered the concentration that presented no growth or a significant lower growth compared to the positive control.

Growth curve

Using the spectrophotometer (Epoch™ 2, BioTech®), a growth curve for the bacteria incubated with all agents separately was made. The plate was incubated at the same conditions in the machine and optic density readings were performed every 2 hours until 48 hours of incubation.

MIC, MBC and growth curve for the clinical isolate

The same procedures to achieve the MIC and MBC were followed as explained previously but using the environmental isolated strain. Only the compound(s) that showed good results for the ATCC was (were) selected for the tests. If more than one presented good effect on the ATCC, but with same patterns of bacterial inhibition, only one would be selected for this part of the experiment.

MIC and MBC of penicillin and challenged bacterium growth curve

The same procedures described above were performed using penicillin for both strains. The concentration ranged from 8 to 0.03125 µg/mL, using serial dilution of 50% from one well to another.

Penicillin and ddNTPs synergism

The *C. pseudotuberculosis* was cultivated in the medium with a mix of penicillin and ddATP, ddCTP or ddGTP. 1 µmol/mL of each nucleotide analogue was tested (25% or less than their MIC) with 50% or 25% of the penicillin. A growth curve, as specified previously, was built and compared with the curves of the substances isolated to determine any difference. Each well with clear medium was cultivated and a resazurin test performed after the 48th hour. First, the synergism would be tested using the ATCC strain and then the environmental strain for the positive results.

Data analysis

For the resazurin test and the BHI agar plate cultures, it was made a binary classification for the results: pink or blue for the first and abundant growth or scarce/no growth for the second. Observational description was made from the growth curves when comparing the positive control with the tests in different concentrations to establish a possible difference in the growth patterns.

3. Results

There was no effect on *C. pseudotuberculosis* ATCC 19410 culture when incubated with the four synthetic nucleotide analogues (ddATP, ddGTP, ddCTP and ddTTP). All wells from concentration of 2 to 0.001 µmol/mL showed high turbidity, presented resazurin reduction observed by the pink colored solution and had no optic density difference when compared to the positive control. It was possible to reach a MIC and MBC for the ddATP, since it was used in higher concentrations, both presenting a value of 4 µmol/mL for the three evaluation methods used (clear solution, blue color on the resazurin test and lower optic density than the positive control). The growth curves had different patterns depending on the concentration and ddNTP added to the medium, showing a right shift for ddATP and ddGTP, which would indicate a delay on the time the bacteria started to grow, and no differences from control to ddCTP and ddTTP. It is possible to confirm the inhibitory effect of the two higher concentrations of ddATP (4 and 8 µmol/mL) by analyzing their curves. Figures 1 to 4 show the growth patterns for each compound. When penicillin was tested, the ATCC 19410 presented a MIC of 0.125 µg/mL and MBC of 0.25 µg/mL.

The first set of results lead to synergism tests of ddATP an ddGTP, which presented a growth delay effect, and one of the two other nucleotide analogues to evaluate if they could possibly potentiate penicillin actions, which was ddCTP. When penicillin was at 25% of its MIC (0.03125 µg/mL) and the ddNTPs at 1µmol/mL (25% of ddATP MIC), none of them presented inhibitory action and the growth curves were similar to the control. But when penicillin was at 50% of its MIC (0.0625 µg/mL), ddATP and ddGTP presented inhibition at the resazurin test, colored blue, and the growth pattern was disrupted due to the inhibitory effect (Figure 5), while the presence of ddCTP did not show difference. Bactericidal action of these synergisms was observed for ddGTP and ddATP by no growth and small amount of colonies formed, respectively, in agar BHI.

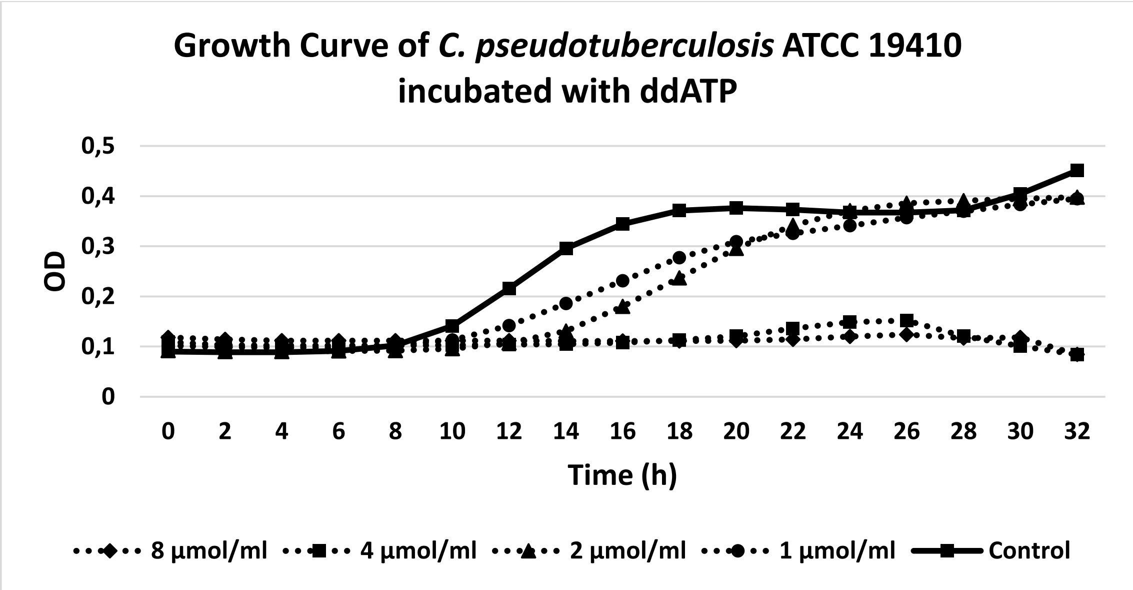

Figure 1. Right shift of the growth curve when using 1 and 2 µmol/mL of ddATP, compared to the control, and inhibition of growth at 4 and 8 µmol/mL. Curves from concentrations lower than 1 µmol/ml were not included due to high similarity with the control. From 32 to 48 hours, the OD did not present variations. OD=optic density.

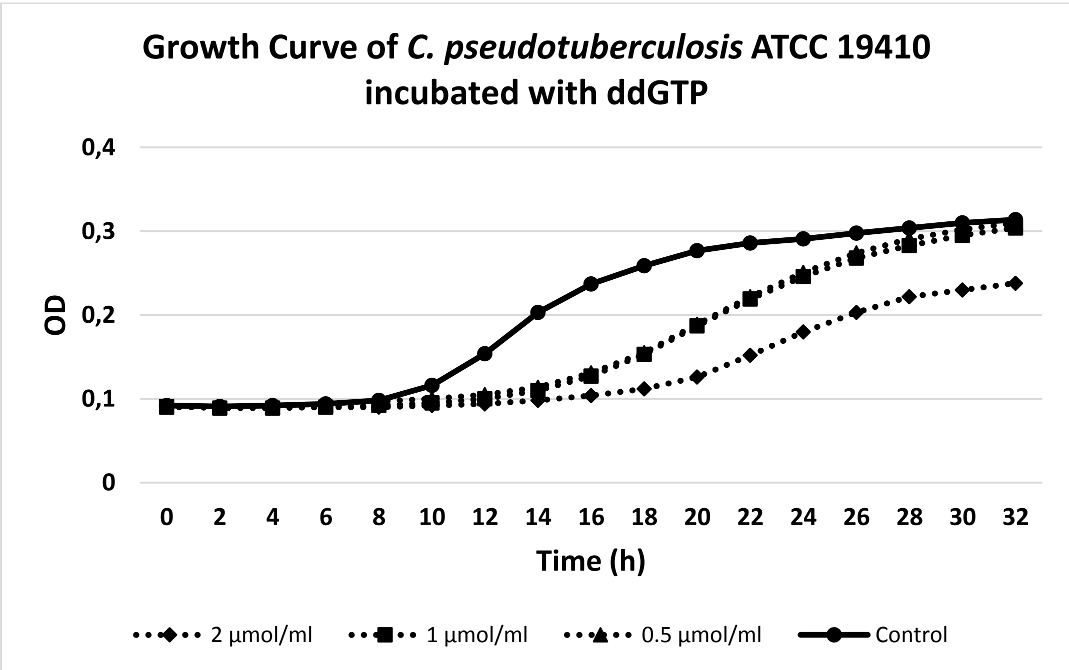

Figure 2. Right shift of the growth curve when using 0.5, 1 and 2 µmol/mL of ddGTP, compared to the control. Curves from concentrations lower than 0.5 µmol/ml were not included due to high similarity with the control. From 32 to 48 hours, the OD did not present variations. OD=optic density.

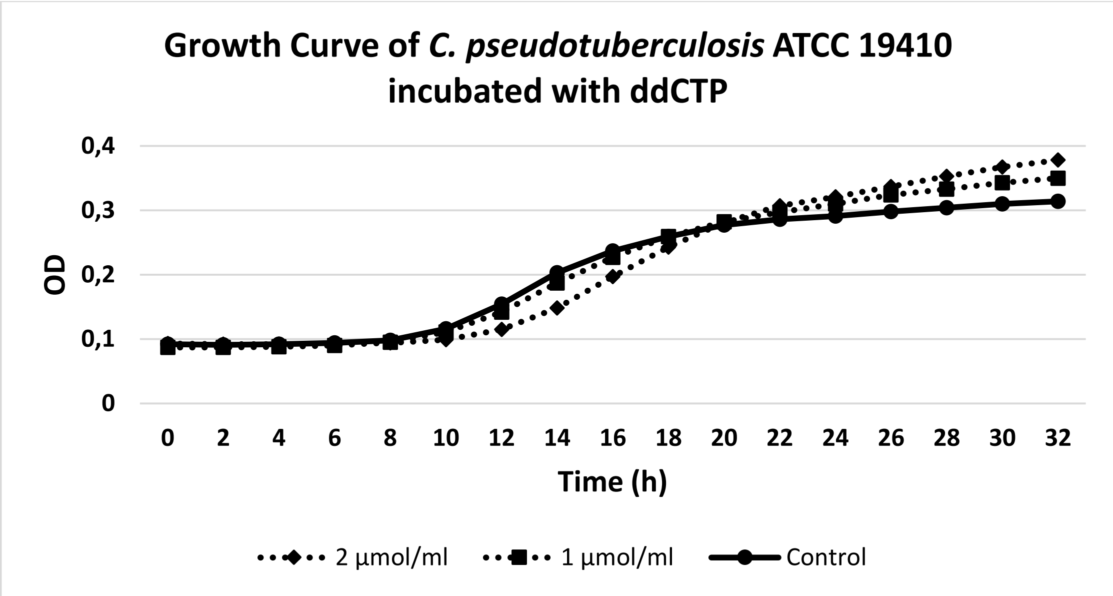

Figure 3. No difference observed among the growth curves when using different concentrations of ddCTP. Curves from concentrations lower than 1 µmol/mL were similar to the ones presented. From 32 to 48 hours, the OD did not present variations. OD=optic density.

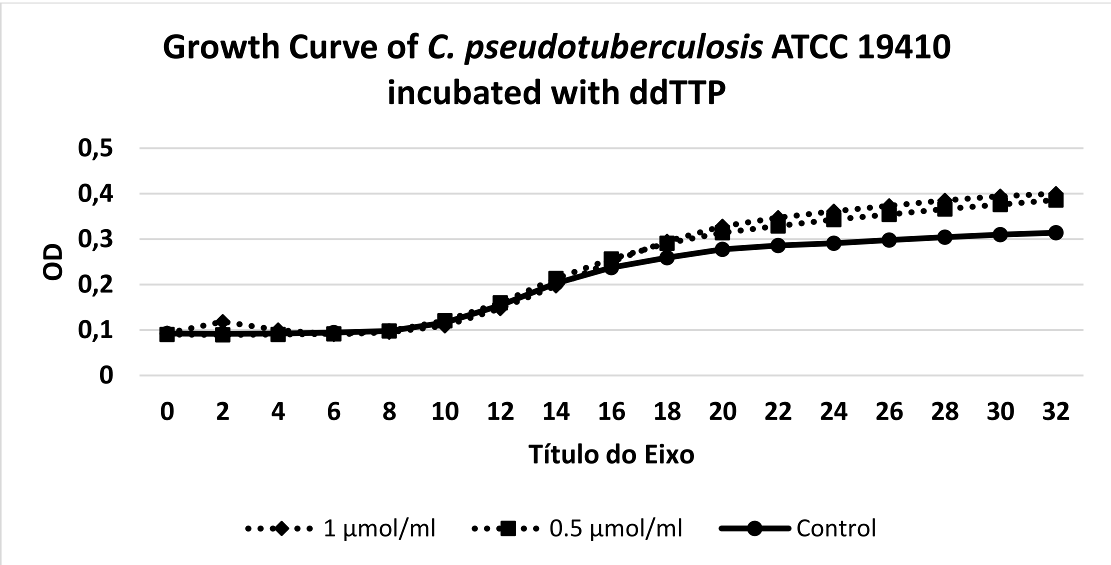

Figure 4. No difference observed among the growth curves when using different concentrations of ddTTP. Curves from concentrations lower than 0.5 µmol/mL were similar to the ones presented. From 32 to 48 hours, the OD did not present variations. OD=optic density.

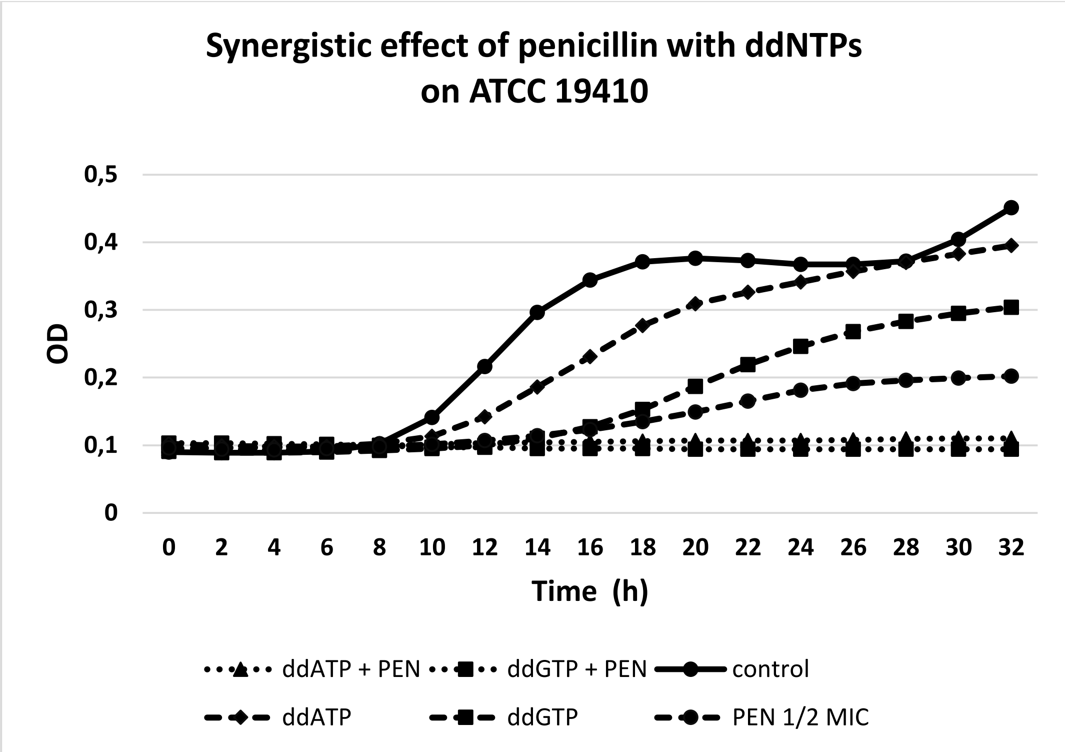

Figure 5. The ddNTPs curves (dashed lines) present a right shift compared to the control. PEN is at 50% of its MIC, not inhibiting total bacterial growth, but only partially. *C. pseudotuberculosis* ATCC 19410 growth was completely inhibited by 50% of PEN MIC with ddATP and ddGTP. From 32 to 48 hours, the OD did not present variations. Nucleotide analogues were at 1 µmol/ml. PEN, penicillin; OD, optic density.

With a MIC and MBC values and positive synergism with penicillin, ddATP was tested in the clinical isolate of *C. pseudotuberculosis*. This strain had a penicillin MIC and MBC of 0.25 µg/mL. Despite presenting a ddATP MIC higher than 4 µmol/mL, this concentration showed a well characterized growth delay effect, when compared with the positive control (Figure 6).

Synergism was tested with 50 and 25% of penicillin MIC (0.0625 and 0.03125 µg/mL) and less than 25% of ddATP MIC (1 µmol/mL). The resazurin and growth curve (Figure 7) evaluations presented inhibitory effect and a cultured agar BHI petri dish showed bactericidal action of the two mixtures by absence of *C. pseudotuberculosis* colonies.

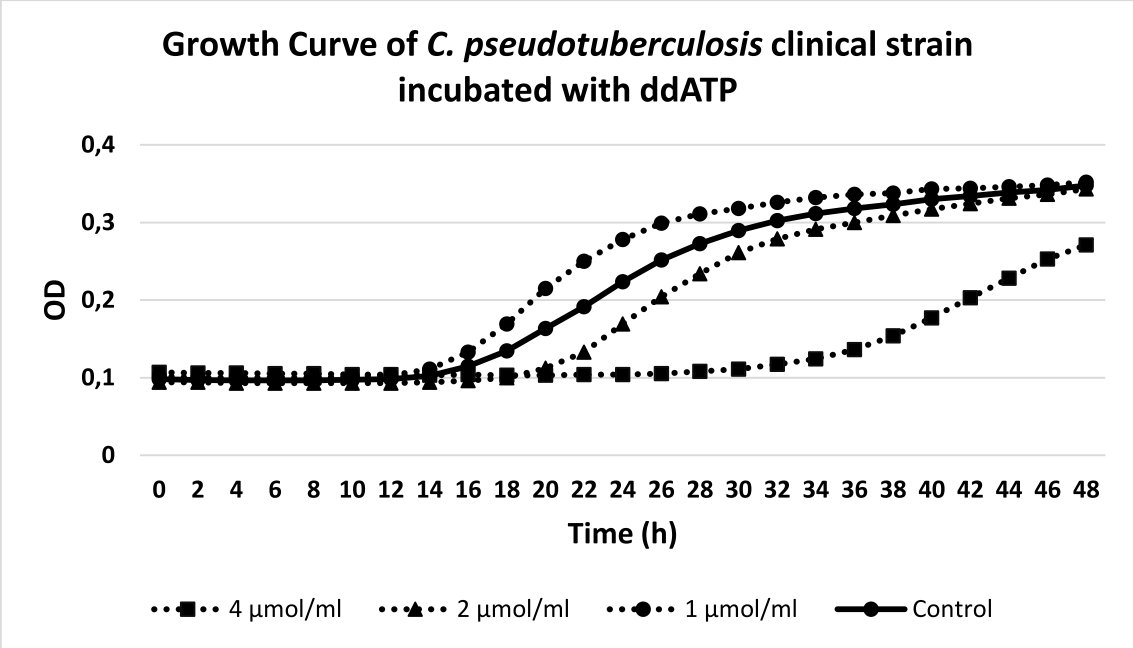

Figure 6. Right shift of the growth curve when using 1, 2 and 4 µmol/mL of ddATP, compared to the control. OD=optic density.

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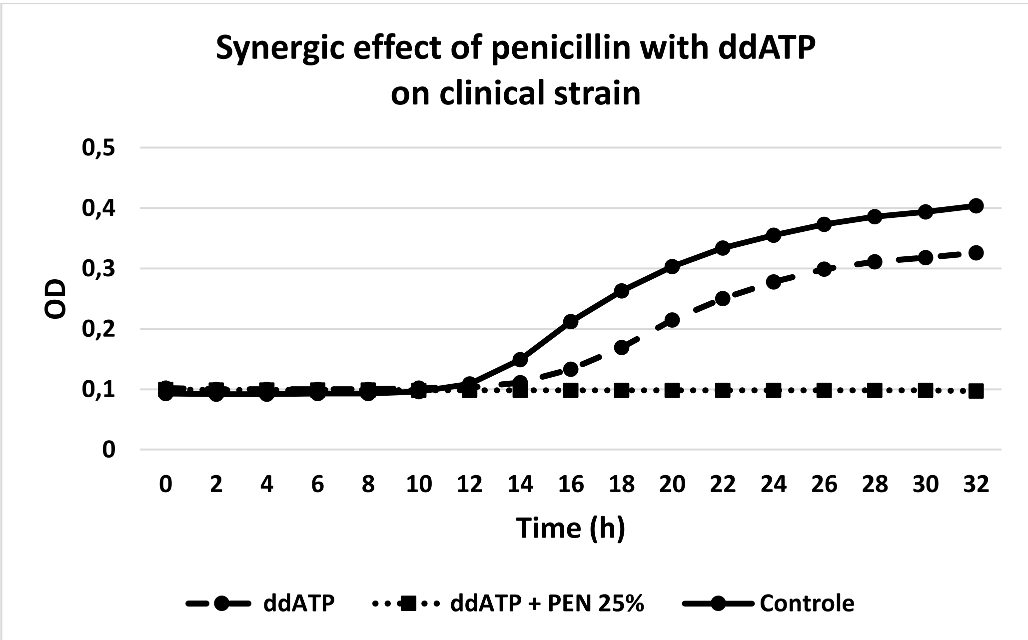

Figure 7. ddATP delayed *C. pseudotuberculosis* growth (dashed curve) compared with the positive control. Combination of ddATP and PEN at 25% of its MIC inhibited growth (dotted line). From 32 to 48 hours, the OD did not present variations. Nucleotide analogues were at 1 µmol/mL. PEN, penicillin; OD, optic density.

4. Discussion

The antibacterial effect presented by ddATP and ddGTP alone or combined with penicillin on *C. pseudotuberculosis* strains demonstrate great importance for veterinary studies of caprine and ovine caseous lymphadenitis, which can be clinically and/or surgically treated, but with possibilities of recurrence or non-effectiveness of the conventional therapeutic methods [9,10,11,12]. Humans treated for *Corynebacterium pseudotuberculosis* infectious also exhibits hardships to achieve total healing [18], leading to possible uses of these synthetic compounds in public health.

The delay effect on bacterial growth evidenced by the right shift on the curves shows an antimicrobial action of the ddATP and ddGTP on *C. pseudotuberculosis* ATCC 19410, besides the MIC and MBC of 4 µmol/mL for ddATP. Moreover, ddATP presented a well characterized delay effect on environmental strain at 4 µmol/mL, indicating a higher MIC than the ATCC. Fourteen other bacteria species were susceptible to ddATP, ten presenting a MIC lower than 2.5 µmol/mL and four ranging from 6 to 13.5 µmol/mL [17], suggesting an intermediate effect on *C. pseudotuberculosis*. A growth curve for *E. coli* challenged with ddATP was compatible to the ones presented in this study, but in different times and concentration: *E. coli* suffered bacteriostatic action with 0.1 µmol/mL of ddATP and bactericidal with 0.3 and 1 µmol/mL within the first 60 and 180 minutes, respectively, faster and in lower concentrations than *C. pseudotuberculosis* [16].

Synergism of nucleotide analogues were studied for treatment of diverse illnesses, but not including bacterial infections. Therapeutic protocols for hepatitis C and acquired immunodeficiency syndrome (AIDS) include more than one drug, the main ones from nucleotide analogues class, some with the same mechanism of action of ddNTPs [19,20]. Neoplasm treatments also include the same class of medication and can combine various compounds [21]. Although no available result of ddNTPs with antibiotics against bacteria was found, our studies indicate a possible positive synergism of penicillin with ddATP and ddGTP, which is well characterized with the environmental strain, showing a potentiation effect by using less than 50% of the MIC of each compound.

The mechanism of action of the ddNTPs would be the inhibition of DNA-polymerase function terminating the duplication of DNA on the middle of the process by blocking the link of the subsequent normal nucleotide, without correction due to saturation of this compound, as seen for other nucleotide analogues [22] and according to the principles of DNA sequencing utilizing ddNTPs [23]. As only purine analogues presented antimicrobial action on *C. pseudotuberculosis*, it suggests a higher prevalence of adenosine and guanine as part of important survival genes of the pathogen.

5. Conclusion

Different therapy options for *C. pseudotuberculosis*-induced infections are important when considering the potential hazard of the pathogen for small ruminants and public health issue. The present study reports for the first time *in vitro* effects of ddNTPs in *C. pseudotuberculosis* strains, showing action of ddATP and ddGTP against this pathogen. These findings represent a further use in clinical trials and alternative therapeutic approach, in addition to the synergism action with penicillin, a new combination that can be used with positive results in other bacteria.

6. Acknowledgement

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2. Trabalho científico 2

Trabalho a ser submetido para a revista Microbial Pathogenesis (ISSN: 0882-4010, Elsevier). Site da revista: https://www.journals.elsevier.com/microbial-pathogenesis. As normas para submissão de manuscrito podem ser encontradas no link: https://www.elsevier.com/journals/microbial-pathogenesis/0882-4010/guide-for-authors.

Natural compounds *in vitro* potentials as new treatment isolated or in synergism with penicillin against *Corynebacterium pseudotuberculosis*

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Highlights

- Cinnamaldehyde and propolis bactericidal action against *C. pseudotuberculosis*;
- Low efficiency of thymol, carvacrol and eugenol against *C. pseudotuberculosis;*
- Synergistic effect of propolis and cinnamaldehyde with penicillin.

Abstract

Corynebacterium pseudotuberculosis infections are common in small ruminants, characterized by lymphadenitis. Clinical and surgical treatments are ineffective, in addition to represent a public health issue. New therapeutic methods with novel antimicrobial agents combined or not with already used antibiotics are important to create better perspectives on how to manage this disease. *In vitro* tests are the first step to determine the possible use of these compounds. Propolis, cinnamaldehyde, eugenol, carvacrol and thymol are substances from essential oils that showed antibacterial action against multiple agents. In the present study, these five substances were added to *C. pseudotuberculosis* cultures to determine their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) and observe if they would present synergism with penicillin. Cinnamaldehyde and propolis exerted antimicrobial action and also presented a positive synergism with penicillin. The results suggest possible use of the two natural compounds with positive effect as an alternative option for treatment of this infection in animals.

Keywords: corynebacteria, cinnamaldehyde, propolis, antimicrobial effect, essential oils.

1. Introduction

Corynebacterium pseudotuberculosis is a gram positive pleomorphic bacteria [1] known for causing caprine and ovine caseous lymphadenitis. The main virulence factors of the pathigen are the lipid surface with mycolic acid protecting from macrophages and the production of exotoxin phospholipase D, which leads to higher vascular permeability, inflammation and necrosis [2]. Clinical signs of *C. pseudotuberculosis*-induced infections are regional lymphnodes swelling and subcutaneous or lymphnodes abscess formation [3,4]. Culture of caseous content of abscesses, inoculation in laboratory animals, molecular detection of bacterial DNA and serological tests are methods recommended to diagnosis the disease [5]. The early clinical and/or serological diagnosis of positive animals and further removal from the flock is the best control method nowadays [6,7], despite multiples vaccines available [8].

Treatment options are surgical removal of abscesses, long duration antibiotic therapies (4 to 6 weeks) or both, although present low efficacy or high risks of abscesses relapse [9,10,11,12]. In this scenario, new treatment options for caseous lymphadenitis are necessary in order to prevent high economic and genetic losses in ovine or caprine production system, making natural compounds, associated or not with conventional antibiotics, potential alternatives.

Propolis is a mix of wax, resin, balsams, volatile oils and some impurities synthetized with secretions of trees, leaves, flowers and pollen by bees [13]. It has shown various biological activities, such as immunomodulatory, anti-inflammatory, anti-neoplasm and antimicrobial [14,15,16,17,18,19]. The antibacterial action has shown to vary according to the extract tested (from different places, seasons and plant resources) and the bacterial species. *Staphylococcus aureus* and *Escherichia coli* presented susceptibility in a MIC and MBC of 2.74 and 5.48 mg/mL, respectively [20] while a different extract showed a MIC of less than 0,1 mg/mL for *S. aureus* and less than 0,38 mg/mL for *E. coli* [21]. Moreover, Dziedzic et al. (2013) [22] proved streptococcic mutant groups and *Lactobacillus* spp. to have MICs of 1.10 and 0.70 mg/mL and MBCs of 9.01 and 5.91 mg/mL, respectively.

The essential oil from cinnamon bark has the cinnamaldehyde as an important part of its composition. Besides hypoglycemic, hypolipemic, immunomodulatory and antifungal activities [23,24,25], it showed antibacterial activity against multiple species with MIC values varying from 0,78 to 650 µg/mL, depending on the bacterial species and strain [23,26,27].

Eugenol is another compound from clove extracts with important biological properties including anti-inflammatory, anti-convulsing, vasodilator, antioxidant, anesthetic and analgesic, anti-neoplasm, anti-parasitic and antifungal [28,29,30,31,32,33]. When incubated with bacteria, proved to disrupt de plasmatic membrane leading to a growth inhibition and bactericidal effects at minimum concentrations of 12.5 µg/ml and 25 µg/mL [34]. Bacterial resistance was not observed by testing subsequent cultures after exposure to eugenol [26] and, associated with multiple antibiotics, presented a positive synergic action [35].

Oregano presents extracts with multiple substances, including thymol and carvacrol. Therapeutic and agricultural properties were attributed to the essential oil as a whole or just one of these two constituents: antifungal, herbicide, insecticide, acetylcholinesterase inhibitor, antioxidant and anti-neoplasm [36,37,38]. The capacity of causing a membrane flaw to bacteria affects the cell ionic and acid-base balance granting these natural compounds an anti-bacterial effect [39,40]. Nevertheless, a biofilm formation inhibition and destruction were observed [41].

This research aims on the detection of therapeutic potential of these five natural compounds against *in vitro* cultures of *C. pseudotuberculosis* and to investigate a possible synergic action when in association with penicillin.

2. Material and Methods

Institutional Animal Care and Use Committee approval

The study was in accordance with the policies of the Institutional Animal Care and Use Comitte (CEUA 0118/2019) of São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, Botucatu/SP, Brazil.

Corynebacterium pseudotuberculosis isolates

A well-characterized and sequenced strain (ATCC 19410) was received from the Institute of Biological Sciences of Federal University of Minas Gerais and three strains were isolated from goats abscesses at the Microbiology Laboratory of the School of Veterinary Medicine and Animal Science from UNESP, Botucatu. They were stocked in a glycerinated brain and heart infusion (BHI) broth at -20°C.

Natural compounds and penicillin

The propolis extract was from the bacteriology laboratory of the Microbiology and Immunology Department (Bioscience Institute of Botucatu, UNESP – Botucatu, SP). Cinnamaldehyde (> 95% pure), eugenol, carvacrol and thymol were purchased from

Sigma-Aldrich (St. Louis, MO) and the penicillin used was Aricilina® (Blau Farmacêutica S.A.; Cotia, SP).

Minimum inhibitory concentration (MIC) of natural compounds

Propolis, cinnamaldehyde, thymol, carvacrol and eugenol were diluted into a 2000 µg/mL solution and sub-dilutions were made from 1000 to 100 µg/mL, decreasing 100 µg/mL in each well of a 96 well plate by using Muller Hinton (MH) broth with 0.2% of Tween 80 in a volume of 100 µL. Cinnamaldehyde was tested in three lower concentrations (50, 25 and 12,5 µg/mL). *C. pseudotuberculosis* colonies were incubated in BHI agar and dissolved into 8.5% saline solution until turbidity reached the 0.5 McFarland standard and then added to the MH broth with 0.2% Tween 80 for a bacterial concentration of 1.5 x 10^6 bacteria/mL. Then, 100 µL of this final solution was added to each well with the compounds, resulting in a final volume of 200 µL/well. Positive and negative controls were made with the same amount of bacteria, for the positive, or no bacteria, for the negative, and final volume as the samples. The plate was incubated at 37°C on a shaking platform (220 rpm) in aerobic conditions during 48 hours. Two evaluations were made: first, a subjective turbidity evaluation of each well; second, a resazurin reduction test. 50 µL of 0.05% resazurin was added into each well, incubated for 2 hours at the same previous conditions and the solution final color was observed, where pink indicated the presence of bacterial growth and blue indicated growth inhibition. The comparison of the two results gave the MIC of these compounds. The experiment was carried out in duplicates beginning with the ATCC strain, followed by the clinical strains if the MIC for the ATCC was 1000 µg/mL or lower.

Minimum bactericidal concentration (MBC) of natural compounds

Before the resazurin test, the wells with no apparent growth that presented clear solution were cultivated in a BHI agar petri dish using calibrated handles, as well as the positive control. Incubation conditions were the same as described previously. The MBC was considered the concentration that presented no growth or a significant lower growth compared to the positive control. The MBC also was performed in duplicates beginning with the ATCC strain and then testing the environmental strains with the substances with positive results.

MIC and MBC of penicillin

The same procedures were performed using penicillin. The concentration ranged from 8 to 0.03125 µg/mL, using serial dilution of 50% from one well to another. They were carried out in duplicates.

Penicillin and natural compounds synergism

C. pseudotuberculosis was cultivated in the medium with a mix of penicillin and the natural compounds that presented MICs lower than 1,000 µg/mL. Concentrations were 50%, 25% and 12.5% of the MIC of each natural compound together with 50%, 25% and 12.5% of the MIC of penicillin. Each well with clear medium was cultivated and a resazurin test performed after the 48th hour. This stage of the experiment was carried out in triplicates.

Data analysis

The resazurin test and the BHI agar plate cultures had an observational description using a binary classification for the results: pink or blue for the first and abundant growth or scarce/no growth for the second.

3. Results

The susceptibility of *Corynebacterium pseudotuberculosis* ATCC 19410 varied for each natural compound. It showed to be resistant to the concentration of eugenol, thymol and carvacrol suggested, therefore they were not incubated with the environmental strains. A high sensibility was observed when exposed to cinnamaldehyde when compared to propolis, but both were able to inhibit growth and kill the bacteria within the concentrations proposed. Cinnamaldehyde and propolis continued exhibiting a similar antibacterial action when tested with the clinical isolates. The penicillin showed great effect on all *C. pseudotuberculosis* strains. The results of the MICs and MBCs for each strain are shown in Table 1.

Table 1. MIC and MBC values for each compound tested in the different strains.

		ATCC (19410)	**Strain 1**	**Strain 2**	**Strain 3**
Propolis	**MIC (µg/mL)**	900	800	800	700
	MBC (µg/mL)	1,000	900	1,000	900
Cinnamaldehyde	**MIC (µg/mL)**	100	200	200	200
	MBC (µg/mL)	200	200	200	200
Eugenol	**MIC (µg/mL)**	>1,000	NT	NT	NT
	MBC (µg/mL)	>1,000	NT	NT	NT
Thymol	**MIC (µg/mL)**	>1,000	NT	NT	NT
	MBC (µg/mL)	>1,000	NT	NT	NT
Carvacrol	**MIC (µg/mL)**	>1,000	NT	NT	NT
	MBC (µg/mL)	>1,000	NT	NT	NT
Penicillin	**MIC (µg/mL)**	0.125	0.25	0.25	0.25
	MBC (µg/mL)	0.25	0.25	0.25	0.25

NT, non-tested.

The independent results led to the synergism study of penicillin with propolis and cinnamaldehyde. The combination of penicillin at 0.0625, 0.03125 and 0.015625 µg/mL with propolis at 450, 225 and 112.5 µg/mL or cinnamaldehyde at 50, 25 and 12.5 µg/mL were tested on ATCC 19410, a total of 9 combinations for each natural compound. There was no synergic effect between penicillin and cinnamaldehyde against the *C. pseudotuberculosis* demonstrated by the reduction of the resazurin by the bacteria transforming the solution into pink color. Propolis with penicillin exhibited a positive synergism when the antibiotic was used in 50% the concentration of its MIC (0.0625 µg/mL) and the bee product in 50%, 25% and 12.5% of its MIC (450, 225, 112.5 µg/mL, respectively). However, when the concentration of penicillin was lower, there was bacterial growth in all wells independently of the amount of propolis. The determinations of bacterial growth presence or absence were the same for turbidity evaluation and resazurin test for the propolis and cinnamaldehyde tests. Bactericidal effect of wells with no bacterial growth was evaluated through culture on BHI agar petri dishes together with the positive control. The comparison among the culture of wells containing penicillin at 0.0625 µg/mL and propolis in all three concentrations demonstrated a positive bactericidal action in all triplicates with the three different concentration of propolis.

The clinical strains were submitted to the same synergism tests using 50, 25 and 12.5% of the MIC of each compound and combining penicillin with propolis or cinnamaldehyde. All three strains demonstrated susceptibility to both mixtures at various concentrations combined, proving a potentiation synergism effect of both natural compounds with penicillin. Table 2 summarizes the synergism tests for all strains.

Table 2. Interaction of penicillin with propolis and cinnamaldehyde in different concentrations.

ATCC 19410				Strain 1			
	Pe.50%	Pe.25%	Pe.12.5%		Pe.50%	Pe.25%	Pe.12.5%
Po.50%	+	-	-	Po.50%	+	+	+
Po.25%	+	-	-	Po.25%	+	-	-
Po.12.5%	+	-	-	Po.12.5%	+	-	-
C.50%	-	-	-	C.50%	+	+	+
C.25%	-	-	-	C.25%	+	+	-
C.12.5%	-	-	-	C.12.5%	+	-	-
Strain 2				**Strain 3**			
	Pe.50%	Pe.25%	Pe.12.5%		Pe.50%	Pe.25%	Pe.12.5%
Po.50%	+	+	+	Po.50%	+	+	+
Po.25%	+	-	-	Po.25%	+	+	-
Po.12.5%	+	-	-	Po.12.5%	+	+	-
C.50%	+	+	+	C.50%	+	+	+
C.25%	+	+	-	C.25%	+	+	-
C.12.5%	+	-	-	C.12.5%	+	-	-

The percentages presented are the concentrations of each product in relation to their MIC. Pe, penicillin; Po, propolis; C, cinnamaldehyde; +, presence of synergism; -, absence of synergism

4. Discussion

Clinical treatment of caprine and ovine caseous lymphadenitis has been performed, although with low efficiency, mainly because of pyogranulomatous reaction. Surgical lymph nodes removal can be performed and combined with antibiotics therapies, but still presenting high chances of recurrence [9,10,11,12]. Humans infected by *Corynebacterium pseudotuberculosis* present signs of caseous lymphadenitis, pneumonia, and necrotizing lymphadenitis. The disease is treated with antimicrobials associated with surgical removal of affected lymph nodes chain or drainage of caseous/purulent material, but can present distress in the total healing of the illness [42,43,44]. A new perspective on treating this infection could be observed with our results.

Although the ability of *Corynebacterium* spp. to biotransform the eugenol in metabolites suitable for pharmaceutical and food industries was reported [45,46], indicatives of antibacterial effect of this clove extract against this species was not found. Our results show high MIC and MBC values (more than 1,000 µg/mL) when compared to *Salmonella typhi* to concentrations of 12.5 µg/mL and 25 µg/mL [34] suggesting a resistance of *C. pseudotuberculosis* to it.

Besides eugenol, *C. pseudotuberculosis* was resistant to thymol and carvacrol (MIC and MBC > 1000 µg/mL), both components of the oregano essential oil. Studies using different extracts with these two substances in the composition proved the existence of susceptibility to this essential oil, but no MIC values could be compared to ours since it was not shown for the compounds alone or the tests were carried in diffusion agar discs [47,48,49,50]. All those observations can indicate a possible effect of thymol and carvacrol in *C. pseudotuberculosis* colonies when associated with the other fractions of the clove essential oil or when used in higher concentrations.

Cinnamaldehyde presented the lower MIC and MBC (ranging from 100 to 200 µg/mL) compared to the other natural compounds, but still higher than penicillin. To our knowledge, there was no information about this compound against corynebacteria nor a possible synergic action with other substances. When compared to its effect on other bacteria, the inhibitory and bactericidal actions required higher concentration for the *C. pseudotuberculosis*. In contrast, other species and groups required amounts ranging from 0.78 to 75 µg/mL [23,26,27]. The antifungal effect was reached on higher concentrations, up to 650 µg/mL [23]. Although presenting antimicrobial activity when incubated alone, no synergism could be observed when associated with penicillin, in any concentration, on the ATCC 19410. Environmental strains were susceptible to combinations lower than 50% of these compounds' MIC, suggesting positive interaction between them and higher sensibility comparing to the ATCC.

C. pseudotuberculosis already presented susceptibility to honey - similar to tetracycline - and the presence and concentration of propolis were suggested as important factors to achieve it [51]. Other species of this genus were tested with propolis from multiple places. *C. bovis* isolated from mastitis was resistant to this bee product, as well as to various antibiotics [52]. *C. striatum* incubated with a French propolis presented MIC ranging 63 and 90 µg/mL [53], while *C. diphtheria* had a MIC of 50 µg/mL for a Turkish propolis [54]. Our results demonstrate a susceptibility of *C. pseudotuberculosis* to propolis alone, without the necessity of the other honey components, but in higher concentrations (700 to 1,000 µg/mL) when compared to other corynebacteria. The synergic potential of propolis with antibiotics was already reported, but not for the bacterium addressed in this study. In 2003, Stepanovic et al. [55] found a positive synergism effect of propolis with antimicrobial drugs used on various bacteria or fungi, but penicillin was not included. When multiple antibiotics and propolis were mixed together, *S. aureus* was susceptible, demonstrating the existence of positive synergism, although penicillin did not present this interaction [56], differently from another study, which it was possible to notice the synergism of penicillin and propolis mix also on *S. aureus* [57]. The diverging results stated can be due to the use of propolis extracts with different compositions, which can alter its biological properties. Our results corroborates with the existence of a positive synergism between propolis and penicillin, not only with an addition effect, but also as a potentiator. ATCC 19410 was killed with 50% of the antibiotic MIC associated with 50%, or less, of the propolis MIC and the other strains were more susceptible since they presented the same results in addition to growth inhibition by less than 50% of penicillin MIC when propolis was at 50% of its MIC, for strains 1 and 2, or even when penicillin was at 25% of its MIC and propolis was at 25% or less.

5. Conclusion

Considering the barriers encountered for this condition's overcome, finding different treatment options becomes important to both public health and animal production. This is the first report of the use of the natural compounds, propolis and cinnamaldehyde, for treating *C. pseudotuberculosis* infections, turning *in vitro* findings of their positive action isolated and synergic action with penicillin, against this bacterial species, important for further use in clinical trials and possibly therapeutic methods.

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7. References

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CAPÍTULO 3

1. Discussão Geral

Existem diversas possibilidades terapêuticas para o tratamento de linfadenite caseosa caprina e ovina. Tanto tratamentos clínicos quanto cirúrgicos ou a combinação dos dois podem ser realizados. Entretanto, as chances de recidiva ou até a baixa eficácia destes métodos representam um problema para a produção animal (GEZON et al., 1991; WILLIAMSON, 2001; OLSON et al., 2002; GUIMARÃES et al., 2011). As infecções de humanos por *C. pseudotuberculosis* apresentam diferentes manifestações e, assim como nos animais, demonstram ser de difícil cura em alguns casos (PEEL et al., 1997; JOIN-LAMBERT et al., 2006; HEGGELUND et al., 2015). Os resultados apresentados por esta pesquisa podem trazer alternativas para melhor eficiência dos tratamentos atuais.

Valores da MIC para ddATP de 14 espécies de bactérias, diferentes da abordada no presente estudo, variaram de 0,2 a 13,5 µmol/mL, das quais dez abaixo de 2,5 e 4 acima de 6 µmol/mL (BESKID et al., 1981), o que mostra que a MIC encontrada para *C. pseudotuberculosis* ATCC 19410 apresenta valor intermediário (4 µmol/mL), enquanto o isolado clínico possuiria um valor mais alto.

As curvas de crescimento deste microrganismo, desafiado por diferentes compostos, demonstram atraso no início da fase log (alongamento da fase *lag*) nas concentrações mais altas de ddATP e ddGTP que não apresentaram inibição total do crescimento (2, 1 e 0,5 µmol/mL para ATCC e 4 µmol/mL para cepa ambiental). Este padrão de crescimento foi evidenciado com *E. coli*, porém em concentração diferente de ddATP (0,1 µmol/mL) e num período de tempo de apenas 3 horas (DOERING et al., 1966).

Não foram encontrados na literatura relatos de sinergismo de análogos de nucleotídeos com antibióticos contra infecções bacterianas, porém, é demonstrado efeito sinérgico positivo quando um conjunto de medicamentos desta classe é usado contra infecções virais (ZEUZEM et al., 2014; WHO, 2018) ou neoplasias (GALMARINI et al., 2002). Os presentes resultados revelaram que a combinação de penicilina com ddATP e ddGTP, em menores concentrações que suas MICs (50% da MIC da penicilina para ATCC e 25% para cepa ambiental e 25% ou menos da MIC dos ddNTPs), apresentam ação inibitória e bactericida contra *C. pseudotuberculosis*, sugerindo potencial sinérgico entre essas classes de compostos.

Supõe-se que o mecanismo de ação destes compostos sintéticos seja a terminação da síntese de novas fitas de DNA pelo bloqueio da ligação de um novo nucleotídeo após a incorporação do análogo, sem correção desta falha por apresentar-se saturada, resumindose pela inibição da correta atuação da DNA polimerase, já observado em outras drogas (HUANG et al., 1990) e seguindo os princípios do sequenciamento genético que utiliza os mesmos compostos (SANGER et al., 1977). Os motivos pelos quais a ddCTP e a ddTTP não demonstraram efeito sob esta bactéria não podem ser elucidados apenas com base nos resultados do presente estudo. Entretanto, é possível hipotetizar sobre a maior prevalência de adenosina e guanina nos genes importantes para sua sobrevivência, resultando na morte das mesmas quando tais nucleotídeos foram substituídos pelos sintéticos, sendo a confirmação possível por estudos biomoleculares.

Dentre os produtos naturais, *C. pseudotuberculosis* apresentou resistência ao eugenol, carvacrol e timol (MIC>1.000 µg/mL). Testes que determinassem tal valor para este gênero de bactéria não foram identificados na literatura consultada, porém já foi observado efeito bactericida do timol e do óleo essencial do orégano, contendo ambos agentes, em testes *in vitro* (BEZBRADICA et al., 2005; NZEAKO et al., 2006; KUMAR et al., 2011; MILOVANOVIC et al., 2016). Já para o eugenol, estudos apenas demonstravam a biotransformação deste em subprodutos com efeito biológico adequado para indústrias farmacêuticas e alimentícias (TADASA & KAYAHARA, 1983; MISHRA et al., 2013), sem dados quanto ao efeito antimicrobiano em isolados de *Corynebacterium* spp.

O melhor efeito antibacteriano com o composto isolado foi observado com o cinamaldeido, variando entre 100 e 200 µg/mL (CIM e CBM) para os quatro isolados testados. Comparado a outras bactérias, tais concentrações foram relativamente altas, uma vez que as MICs das outras espécies variaram de 0,78 a 75 µg/mL (SANLA-EAD et al., 2010; ALI et al., 2005; OOI et al., 2006), porém podem ser consideradas baixas se a referência for o efeito antifúngico, que chegou a concentrações tão altas quanto 650 µg/mL (OOI et al., 2006). Apesar do efeito bactericida estar evidente neste composto derivado da canela, quando utilizado em concentrações mais baixas em conjunto com a penicilina, também abaixo de seu MIC, não ocorre efeito aditivo nem potencializador para a ATCC 19410. Entretanto, em isolados clínicos, a associação desses compostos em valores menores que suas MICs, até mesmo 25% da MIC de cada um deles, apresentou efeito antimicrobiano, demonstrando o potencial desta combinação de produtos e o fato das cepas ambientais serem mais suscetíveis que a ATCC.

Efeito *in vitro* da própolis já foi descrito em isolados do gênero *Corynebacterium*, sendo que alguns testes comprovaram resistência do organismo a este produto (HEGAZI et al., 2014) e outros mostraram suscetibilidade (BAYRAM et al., 2015; BOISARD et al., 2015), dependendo da espécie, com MICs de 63 a 90 µg/mL. Além disso, testes em associação mostraram sinergismo positivo entre a própolis e antimicrobianos, tanto para fungos quanto bactérias (STEPANOVIC et al., 2003), sendo relatados resultados tanto positivos quanto negativos quando o antibiótico selecionado foi a penicilina (JUNIOR et al., 2005; WOJTYCZKA et al., 2013). Comparando com os resultados do presente estudo, a própolis revelou efeito inibitório e bactericida em concentrações maiores que as mencionadas, variando entre 700 a 1.000 µg/mL, enquanto o efeito sinérgico corroborou com resultados positivos para a interação com a penicilina, necessitando de valores tão baixos quanto 25% da MIC da penicilina e 12,5% da MIC da própolis. Tais variações podem ser relacionadas com a própolis utilizada, que pode variar em sua composição dependendo do local coletado, estação do ano, tipo vegetal usado como base, entre outros fatores.

2. Conclusão Geral

Testes com nove substâncias não utilizadas no tratamento recomendado de infecções pelo *Corynebacterium pseudotuberculosis* possuem importância por representarem possíveis novas opções terapêuticas contra este microrganismo. dois produtos sintéticos (2’3’-dideoxiadenosina e 2’3’-dideoxiguanina) e dois naturais (cinamaldeido e própolis) revelaram ação antibacteriana *in vitro* sob este agente, além de apresentarem efeito positivo quando utilizados em conjunto com a penicilina, antibiótico utilizado para o tratamento clínico de linfadenite caseosa. Tais resultados são a primeira etapa para a utilização desses produtos - combinados ou não - em casos clínicos desta afecção. Testes *in vivo* seriam indicados para confirmar a ação destes compostos, além das doses a serem utilizadas, farmacodinâmica e farmacocinética. Nas infecções por *C.* pseudotuberculosis, as novas opções de tratamento representariam perspectivas de boa eficácia, além de alternativa para casos de resistência aos antimicrobianos convencionais.

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ANEXO 1

Aprovação do projeto pela Comissão de Ética no Uso de Animal

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

fmvz - unesp
Faculdade de Medicina Veterinária e Zootecnia
Campus de Botucatu

ATESTADO

Atesto que o Projeto "INIBIÇÃO IN VITRO DO CRESCIMENTO DE Corynebacterium pseudotuberculosis PELO POSSÍVEL SINERGISMO DE DIDEOXINUCLEOTÍDEOS COM PENICILINA" **Protocolo CEUA 0118/2019** , a ser conduzido por Pedro Negri Bernardino, responsável/orientador José Paes de Oliveira Filho, para fins de pesquisa científica/ensino - encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal - CONCEA.

Finalidade	PESQUISA CIENTÍFICA
Vigência do projeto	24/06/2019 a 01/02/2020
Nome Comum / Espécie / Linhagem	/ /
Raça	
Nº de animais machos	0
Nº de animais fêmeas	0
Nº de animais sexo indefinido	0
Peso médio de animais machos	0
Peso médio de animais fêmeas	0
Peso médio de animais sexo indefinido	0
Idade	ano(s) e 0 mes(es) e 0 dia(s).
Procedência	FMVZ

Projeto de Pesquisa aprovado em reunião da CEUA em 10/07/2019

JOSÉ NICOLAU PRÓSPERO PUOLI FILHO
Presidente da CEUA da FMVZ, UNESP - Campus de Botucatu

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