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FORMAÇÃO DE BIOFILME E MECANISMO DE EFLUXO EM ISOLADOS DE *Rhodococcus equi*

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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FORMAÇÃO DE BIOFILME E MECANISMO DE EFLUXO EM ISOLADOS DE *Rhodococcus equi*

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Dissertação apresentada ao Curso de Mestrado do 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 do grau de **Mestre em Medicina Veterinária.**

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A Deus, que sempre iluminou e abençoou as minhas escolhas.

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Sonho que se sonha só é só um sonho que se sonha só, mas sonho que se sonha junto é realidade.

Prelúdio (Raul Seixas)

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

FORMAÇÃO DE BIOFILME E MECANISMO DE EFLUXO EM ISOLADOS DE *Rhodococcus equi*

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Data e Local de Defesa: Santa Maria, 22 de fevereiro de 2013.

O objetivo do presente estudo foi determinar a ocorrência de dois mecanismos de resistência descritos em micro-organismos, porém, ainda não verificados em isolados de *R. equi* provenientes de amostras de equinos e de solo. O primeiro mecanismo estudado foi a formação de biofilmes em isolados de *R. equi* de amostras clínicas (*n*=41) e fecais (*n*=72) de equinos. Para verificação da formação de biofilmes empregaram-se as técnicas de formação de biofilme em cultura (com e sem a suplementação de glicose) e a microscopia de epifluorescência. Oito isolados formadores de biofilme foram selecionados e submetidos a testes de susceptibilidade frente a três antimicrobianos da classe dos macrolídeos, comumente utilizados no tratamento da rodococose equina. A formação de biofilmes foi observada em 80,5% dos isolados fecais e em 63,4% dos clínicos. Os métodos utilizados neste estudo foram adequados para verificar a formação de biofilmes nos isolados de *R. equi*. No entanto, foi constatado que a glicose é um substrato importante para formação de biofilme em isolados de origem fecal. Nos testes de susceptibilidade observou-se resistência aos antimicrobianos testados apenas quando os isolados de *R. equi* foram desafiados na forma de biofilme. O segundo mecanismo explorado envolveu a pesquisa de sistema de efluxo em isolados de *R. equi* de origem clínica (*n*=30) fecal (*n*=30) e de solo (*n*=30). Neste estudo buscou-se identificar a presença de um gene denominado *req_39680*, o qual foi descrito como codificador de um possível sistema de efluxo, Por fim, avaliou-se a expressão fenotípica de mecanismo de efluxo nos isolados analisados. A presença do gene

req_39680, foi determinada por meio da técnica de reação em cadeia da polimerase (PCR) e a análise da expressão fenotípica de mecanismo de efluxo foi visualizada em ágar contendo brometo de etídeo. Foi evidenciada a presença do gene *req_39680* em 60% dos isolados e a expressão fenotípica em 20%. Nos isolados de origem clínica, um alto índice de correlação entre a presença do gene estudado e a expressão de mecanismo de efluxo foi observada. Ambos os mecanismos estudados estão presentes em *R. equi* e podem estar contribuindo para a crescente resistência aos antimicrobianos, bem como, estar relacionados à sobrevivência de *R. equi* tanto no ambiente quanto no hospedeiro.

Palavras-chave: Biofilmes, Bombas de Efluxo, Resistência, Rodococose.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

BIOFILM FORMATION AND EFFLUX MECHANISM IN *Rhodococcus equi* ISOLATES

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The goal of this study was to determine the occurrence of two resistance mechanisms described in micro-organisms, but not described in *R. equi* isolates from horses and environment samples. The first mechanism studied it was the biofilm formation in *R. equi* isolates from clinical (*n*=41) and fecal (*n*=72) equine samples. In order to verify the biofilm formation it was employed the biofilm-culturing assay (with and without glucose supplementation) and the epifluorescence microscopy method. Biofilm-producers *R. equi* isolates (*n*=8) were selected and analyzed by two in vitro susceptibility tests with three antimicrobial agents belonging to macrolides group and commonly used in equine rhodococcosis treatment. The biofilm formation was observed in 80.5% of fecal and 63.4% of clinical isolates of *R. equi*. The methods used in this study were useful to verify the biofilm formation by *R. equi* isolates. However, the glucose supplementation was an important factor for biofilm formation in fecal samples. Antimicrobial resistance was demonstrated in biofilm antimicrobial susceptibility test. The second mechanism studied included the efflux system in *R. equi* isolates from clinical (*n*=30), fecal (*n*=30) and soil (*n*=30) sources. The

presence of *req_39680* gene, encoding a putative efflux system, was determined by PCR. Phenotypic expression of efflux systems was determined in agar containing ethidium bromide. The *req_39680* gene was detected in 60% of the isolates and the phenotypic expression in 20%. In clinical isolates was observed high correlation between the presence of this gene and the expression of efflux systems. Both mechanisms studied were demonstrated in *R. equi* and may be contributing to increase the antimicrobial resistance, as well as to be associated to the survival of *R. equi* in the environment and host.

Key words: Biofilms, Efflux pumps, Resistance, Rhodococcosis.

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SUMÁRIO

1. INTRODUÇÃO

O Brasil possui o maior rebanho de equinos da América Latina e o quarto mundial, os quais, somados aos muares (mulas) e asininos (asnos) compõe 7,8 milhões de cabeças (FAO, 2013). Esta importante cadeia do agronegócio movimenta a ordem econômica de R$ 7,3 bilhões por ano e é responsável pela geração direta e indireta de 3,2 milhões de empregos (ESALQ, 2006). Na região sul do Brasil, encontram-se muitos haras destinados à criação de equinos com alto padrão zootécnico, sendo a sanidade destes rebanhos de fundamental importância para o sucesso deste tipo de empreendimento. No entanto, devido à produção intensificada, a ocorrência de enfermidades infecciosas tem aumentado, especialmente as do trato respiratório, refletindo em prejuízos econômicos associados à criação de equídeos.

No mundo, a pneumonia é a principal causa de morte em potros (GIGUÈRE et al., 2011). Dentre as enfermidades que acometem esta categoria animal, a rodococose, causada pelo micro-organismo *Rhodococcus equi* (PRESCOTT, 1991), é apontada mundialmente como a doença mais debilitante (BECÚ, 1999; RIBEIRO et al., 2005). No Brasil, dentre as doenças respiratórias, está é a mais importante em potros (RIBEIRO et al., 2005) e em criatórios da região sul do Brasil é a segunda mais prevalente em potros com menos de 6 meses de idade (FREY JR, 2006). As perdas econômicas relacionadas à rodococose equina estão associadas com cuidado médico veterinário, longo período de tratamento e mortalidade dos animais (VENNER et al., 2012). Além disso, esta enfermidade é responsável por complicações secundárias que podem encerrar prematuramente a vida produtiva do animal (AINSWORTH, 1997). Ao mesmo tempo, a rodococose tem importância em saúde publica por representar uma zoonose, acometendo, especialmente, indivíduos imunocomprometidos, idosos e crianças (PRESCOTT, 1991).

Neste capítulo, serão abordadas informações referentes ao micro-organismo *Rhodococcus equi* e enfatizados os aspectos relacionados ao diagnóstico, tratamento, controle e profilaxia da rodococose equina. Por fim, serão apresentados dois importantes mecanismos de resistência de micro-organismos, conhecidos por biofilmes e bombas de efluxo.

1.1 Rodococose equina

Rhodococcus equi pertence ao gênero *Rhodococcus*, incluso na ordem Actinomycetales, juntamente com outros gêneros como: *Corynebacterium*, *Mycobacterium*, *Arcanobacterium* e *Nocardia*, agrupados de acordo com a composição da parede celular (FINNERTY, 1992). *R. equi* é um patógeno intracelular facultativo, apresenta-se sob a forma de cocos ou bacilos pleomórficos Gram-positivos e caracteriza-se por sobreviver e replicar no interior de macrófagos (PRESCOTT, 1991). Em equinos, especialmente potros entre um a seis meses de idade (SELLON, 2001), causa broncopneumonia piogranulomatosa, podendo cursar com entrerocolite ulcerativa, linfadenite mesentérica, osteomielite, artrite purulenta e linfangite ulcerativa (PRESCOTT, 1991). Em outras espécies de produção (bovinos, caprinos e bubalinos) e de companhia (cães e gatos) a doença é rara, ocorrendo sob a forma de enterite, linfadenite, abortamento, mastite, dermatite e piometra (PRESCOTT, 1991). Em suínos o comportamento não é progressivo, apresentando–se como linfadenite com lesões semelhantes às observadas na tuberculose (TAKAI, 1997). Destaca-se, ainda, o potencial zoonótico deste agente. O primeiro relato de caso de infecção humana por *R. equi* ocorreu em 1967 em um paciente severamente imunocomprometido (GOLUB et al., 1967), entretanto, casos da doença em indivíduos hígidos, especialmente crianças e idosos também foram relatados (MACGOWAN & MANGANO, 1991; FARINA et al., 1997). Na última década, *R. equi* foi descrito em humanos, em especial entre os portadores do HIV (vírus da imunodeficiência humana), associado à pneumonia, abscessos pulmonares e infecções sistêmicas (BYRNE et al., 2001; VON BARGEN & HAAS, 2009).

A patogenicidade de linhagens de *R. equi* está intimamente relacionada a uma ilha de patogenicidade formada por dez genes que codificam proteínas associadas à virulência (*virulence associated protein*, Vap*)*. Estes genes também foram denominados *vap* e classificados como: *vapA*, *vapB*, *vapC*, *vapD*, *vapE*, *vapF*, *vapG*, *vapH*, *vapI* e *vapX* (RUSSELL et al., 2004; LETEK et al., 2008). Após, cinco novos membros da família *vap* foram descritos: *vapJ*, *K1*, *K2*, *L*, e *M* (LETEK et al., 2008). O principal antígeno de virulência relacionado à infecção em equinos melhor caracterizado até o momento é a proteína de superfície VapA, codificada pelo gene *vapA* (BYRNE et al., 2001). Em um estudo com camundongos observou-se que isolados *vapA*-negativos não possuem a capacidade de sobreviver e replicar no interior de macrófagos, refletindo em queda acentuada da letalidade (TAKAI et al., 1991). Posteriormente, a importância deste gene foi demonstrada em estudos nos quais se observou que a perda do plasmídeo de virulência que codifica para proteína VapA coincide também com a diminuição da

patogenicidade do *R. equi* para camundongos e equinos (TAKAI et al., 1995; WADA et al., 1997). A partir do conhecimento destes genes, as cepas de *R. equi* passaram a ser classificadas em três tipos (TAKAI et al., 1991): isolados virulentos, caracterizados pela presença da proteína VapA (15 a 17kDa) (RIBEIRO et al., 2005), isolados de virulência intermediária, carreadores da VapB (BYRNE et al., 2001) e avirulentos, que não apresentam plasmídeo relacionado à virulência (RIBEIRO et al., 2005).

Em relação à distribuição dos genes *vap*, o antígeno VapA é encontrado em todos os isolados clínicos de *R. equi* obtidos de potros, bem como em algumas amostras ambientais e de origem humana (TAKAI et al., 1996; COSTA et al., 1999). Entretanto, existem relatos de amostras desprovidas de plasmídeo de virulência ocasionando infecções no homem e animais, especialmente bovinos e caprinos (CANTOR et al., 1998). Todavia, as cepas VapB-positivas, são comumente encontradas em linfonodos submandibulares de suínos e em isolados de humanos imunossuprimidos (TAKAI et al., 1996). Recentemente, também foram descritas em linfonodos de javalis (LARA et al., 2011). Em relação aos isolados avirulentos, os mesmos estão presentes principalmente no solo e em amostras de fezes de equinos saudáveis (RIBEIRO et al., 2005).

O desenvolvimento de patologias respiratórias em potros está intimamente ligado ao tipo de placenta da espécie (epiteliocorial difusa) que atua como uma barreira à passagem de grandes moléculas, como as imunoglobulinas. Desta forma, a transferência passiva de anticorpos via colostro é fundamental para a proteção contra às infecções no período neonatal (LEBLANC et al., 1992). De uma forma geral, a infecção por *R. equi* possui caráter oportunista, pois o período em que os potros desenvolvem pneumonia coincide tanto com o momento de declínio das taxas de anticorpos adquiridos pela imunidade passiva, quanto com a imaturidade do sistema imunológico do animal (GYLES et al., 2010). Os fatores que parecem estar associados ao aumento da ocorrência da doença incluem: alta densidade de animais, sistema de criação e manejo, elevado número de cepas virulentas no ambiente. Além disso, os fatores ambientais como baixa umidade, altas temperaturas, tipo de material encontrado no piso das cocheiras (terra ou concreto) e um pasto com deficiência de nutrientes, também contribuem para o aumento das infecções (COHEN et al., 2005, MUSCATELLO et al., 2007). De acordo com JONES et al. (1997) a morbidade por *R. equi* pode atingir 20% e a mortalidade 80%, sendo que em algumas propriedades este micro-organismo ocorre esporadicamente e em outras é endêmico, resultando em surtos frequentes da doença (TAKAI, 1997).

O diagnóstico definitivo da pneumonia causada por *R. equi* deve basear-se em isolamento e identificação do agente a partir de amostras de aspirado traqueobronquial, especialmente por cultura bacteriológica e na reação em cadeia da polimerase (PCR) identificando o gênero, a espécie e o gene *vapA* (GIGUÈRE, 2001). As análises sorológicas, como por exemplo, a técnica de imunodifusão em gel de ágar, são ineficazes em diagnosticar a infecção por *R. equi*, principalmente porque os anticorpos contra *R. equi* estão normalmente presentes na população (GIGUÈRE, 2001). DEPRÁ (2001) demonstrou que esta técnica não diferencia a resposta ativa de anticorpos entre potros sadios e doentes, propondo o monitoramento clinico diário como forma mais eficaz de diagnosticar precocemente a doença.

Devido à natureza intracelular, a pneumonia por *R equi* é tratada com antimicrobianos lipofílicos durante um prolongado período de tempo (PRESCOTT, 1991). O tratamento geralmente consiste de aplicação combinada de macrolídeos, tais como a eritromicina, associados a rifampicina (GIGUÈRE, 2000). A combinação da eritromicina e rifampicina promove uma ação sinérgica, havendo uma boa penetração em macrófagos e tecidos. Devido a isso, esta opção terapêutica vem sendo utilizada no tratamento da rodococose em potros, gerando uma drástica redução nas taxa de mortalidade (HILLIDGE, 1987, referencia atual). No entanto, a concentração inibitória mínima (CIM) da rifampicina e eritromicina para cepas de *R. equi* isoladas durante os últimos 10 anos têm aumentado (BUCKLEY et al., 2007), e o aparecimento de algumas cepas resistentes aos diferentes antimicrobianos têm sido relatado (TAKAI et al., 1997; ASOH et al., 2003; NIWA et al., 2006). CHAFFIN (2007) sugere que a azitromicina seja o fármaco mais indicada para o tratamento da rodococose, uma vez que ameniza os efeitos indesejáveis observados no tratamento com eritromicina. Além disso, diversos estudos apontam para uma crescente resistência aos macrolídeos envolvendo, basicamente, três estratégias: presença de metilases, atividade de bombas de efluxo e inativação por enzimas (SUTCLIFFE et al., 1996).

O controle e a prevenção de infecções por *R. equi* é difícil devido sobretudo a sua natureza insidiosa que representa um obstáculo à detecção precoce da doença e o isolamento dos animais infectados. É importante salientar que o diagnóstico precoce da enfermidade é imprescindível, pois auxilia na diminuição da ocorrência da doença clínica, da mortalidade e dos custos com o tratamento (MUSCATELLO, 2012). Para TAKAI (1997) e DEPRÁ et al. (2001) a higiene rigorosa, o monitoramento clínico diário e o tratamento do potro enfermo com antimicrobianos específicos constituem as medidas mais eficazes na prevenção da rodococose. O

emprego da vacinação é discutível; no entanto, os potros vacinados parecem estar mais protegidos que os não imunizados (GUIGUÈRE, 2001). NEWTON (2007) sugere que em locais onde existam fatores de risco sejam adotadas práticas de biossegurança e/ou biosseguridade a fim de limitar a concentração do micro-organismo no ambiente e, consequentemente, diminuir os riscos de contaminação por *R. equi* .

1.2 Biofilmes

Biofilmes são considerados um estágio de desenvolvimento bacteriano (O' TOOLE et al., 2000). Por definição, compreendem colônias microbianas aderidas a uma superfície e, normalmente, embebidas em uma matriz polimérica extracelular (MPE) (VERMELHO et al., 2007). Nesta estrutura, as bactérias passam da forma planctônica para a vida séssil; além disso, as modificações na fisiologia e resistência aos diversos tipos de estresse são evidentes nos micro-organismos formadores de biofilmes (O' TOOLE et al., 2000). Cabe ressaltar que, é possível estimar mais de 90% da vida microbiana ocorrendo na forma de biofilmes (CHARAKLIS & MARSHALL, 1990).

A formação de biofilmes parece ser influenciada pelo processo de sinalização célula-célula, conhecido como *quorum sensing*, que envolve a produção e a detecção de moléculas sinalizadoras extracelulares chamadas autoindutores (SIFRI, 2008). O desenvolvimento de biofilmes bacterianos ocorre, basicamente, com a adesão da célula a uma superfície. Após este evento, inicia-se o processo de colonização seguido da formação de microcolônias, que, na maioria dos casos, diferenciam-se em macrocolônias, e posteriormente, havendo a maturação, a ruptura e a dispersão do biofilme (VERMELHO et al., 2007).

A primeira observação registrada de biofilmes foi realizada por Arthur T. Henrici em 1933 (HENRICI, 1933); porém, apenas em 1978 (COSTERTON et al., 1978) sua natureza abrangente e predominante foi devidamente constatada. Posteriormente, observou-se, em certas infecções, que a cronicidade e a resistência a agentes antimicrobianos estavam intimamente ligadas ao crescimento bacteriano na forma de biofilmes (VERMELHO et al., 2007). Estudos relatam que infecções associadas com a formação de biofilmes são 10 a 1000 vezes mais resistentes aos agentes antimicrobianos (OLSON et al., 2002; CERI et al., 2010). Este aumento da resistência parece estar relacionado a diversos fatores, como por exemplo: a barreira composta de MPE; aos diferentes estados metabólicos das bactérias (JACQUES et al., 2010); e, ao fato dos

biofilmes serem, muitas vezes, compostos de inúmeras espécies bacterianas, aumentando as opções de proteção contra a ação de antimicrobianos (HALL-STOODLEY & STOODLEY, 2009).

A capacidade de estabelecer biofilmes não está restrita a um grupo específico de micro-organismos, sendo que, sob as condições ambientais adequadas, todos os micro-organismos são capazes de desenvolverem-se na forma de biofilmes (LASA et al., 2005). Aproximadamente 75% das infecções em humanos são causadas pela formação e persistência de biofilmes microbianos (RICHARDS & MELANDER, 2009). Diante do significativo envolvimento dos biofilmes nas infecções de humanos, é muito provável que este mecanismo de resistência também esteja determinando uma grande variedade de infecções em medicina veterinária (PARSEK & SINGH, 2003; CLUTTERBUCK et al., 2007, JACQUES et al., 2010).

1.3 Bombas de efluxo

O efluxo de antimicrobianos como mecanismo de resistência foi primeiramente reconhecido para a tetraciclina, em *Escherichia coli*, no fim da década de 70 (BALL et al., 1977) e início da década de 80 (McMURRY et al., 1980). Esses sistemas existem em bactérias Gram-negativas, Gram-positivas e eucariotos (WEBBER & PIDDOCK, 2003) e bombeiam os compostos tóxicos à célula de maneira ativa utilizando o gradiente de prótons ou a hidrólise de ATP como força motriz (WEBBER & PIDDOCK, 2003). Desta forma, as bombas de efluxo são proteínas de extrusão de compostos tóxicos, divididas em cinco famílias: i. Superfamília de facilitadores principais (*Major facilitator superfamily* - MFS), ii. Superfamília de ligação adenosina-trifosfato (*Adenosine triphosfate-binding cassete* - ABC), iii. Família de resistência a múltiplos fármacos menores (*Small multidrug resistance family* - SMR), iv. Superfamília de divisão celular, nodulação e resistência (*Resistance-nodulation-cell division superfamily* - RND) e v. Família de extrusão de componentes tóxicos e múltiplos fármacos (*Multidrug and toxic compound extrusion family* - MATE) (KUMAR & SCHWEIZER, 2005).

As bombas de efluxo compreendem proteínas capazes de expulsar múltiplos fármacos, incluindo diferentes classes de antimicrobianos. Este fato constitui um sério problema para o tratamento de pacientes com enfermidades infecciosas. Além disso, este mecanismo pode estar associado tanto à resistência antimicrobiana intrínseca quanto a adquirida em infecções humanas e animais (PUTMAN et al., 2000).

Em virtude da importância dos mecanismos de resistência acima apresentados e da crescente resistência antimicrobiana observada frente aos isolados de *R. equi*, este estudo teve como objetivo elucidar a ocorrência dos mecanismos de formação de biofilmes e de bombas de efluxo em isolados brasileiros de *R. equi*. Para tanto, no primeiro artigo, abordou-se a formação de biofilmes através do emprego de duas técnicas fenotípicas e avaliou-se a influência deste mecanismo na resistência a três antimicrobianos comumente utilizados no tratamento da rodococose equina. No segundo artigo, buscou-se identificar o gene *req_39680*, relacionado a um possível sistema de efluxo, bem como verificar a sua correlação com a expressão fenotípica de mecanismo de efluxo em isolados de *R. equi* de diferentes origens.

2. ARTIGO I

(Artigo a ser submetido ao periódico Microbiology)

BIOFILM DEVELOPMENT AND SUSCEPTIBILITY OF PLANKTONIC AND BIOFILM *Rhodococcus equi*

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Summary

This study aimed to evaluate the biofilm formation and the activity of antimicrobials against planktonic and biofilm *R. equi*. The biofilm formation was evaluated in 113 *R. equi* isolates of clinical and fecal equine samples by both biofilm-culturing (BC) with and without additional glucose and epifluorescence microscopy (EM) methods. The correlation indexes among the methods were also established. Subsequently, eight *R. equi* isolates biofilm-producers were evaluated by two antimicrobial susceptibility tests using azithromycin, clarithromycin and erythromycin. It was verified 74.3% (84/113) biofilm-positive in at least one of the methods tested. Biofilm-producers *R. equi* were observed in fecal (80.5%) and clinical (63.4%) isolates. In clinical *R. equi* isolates by BC supplemented with glucose (BCG) and EM assays demonstrated the highest correlation index (0.56). However, the fecal *R. equi* isolates had a correlation index of 0.52 by the same both methods. The susceptibility profile observed in the planktonic *R. equi* cells was susceptible to intermediate for all antimicrobials tested. Moreover, the established biofilms *R. equi* were no eradicated with the same antimicrobials tested. Additionally, it was observed that biofilm *R. equi* cells were more reduced in the presence of clarithromycin than azithromycin and erythromycin. *R. equi* isolates from clinical and fecal samples are able to form biofilm; however, the biofilm formation by fecal *R. equi* isolates is more frequent than in clinical isolates. BCG and EM are adequate for screening the biofilm formation specially in fecal *R. equi* isolates. The addition of 0.25% glucose does not appear to influence the biofilm formation by *R. equi* isolates from clinical samples. The antimicrobials susceptibility tests demonstrate the great resistance of

biofilm *R. equi* cells. Future biofilm researches are necessary to evaluate epidemiology and pathogenesis of biofilm-producers *R. equi*.

Keywords: biofilm formation, DAPI, microtiter, rhodococcosis, *Rhodococcus equi*

INTRODUTION

Rhodococcus equi is a facultative intracellular (Prescott, 1991) and telluric (Prescott, 2004) pathogen. It is also the etiologic agent of equine rhodococcosis, a disease that typically affects 3-week- to 6-month-old foals (Giguère & Prescott, 1997). This bacterium has worldwide distribution and is frequently widespread in the environment, especially, in horse-breeding farms (Takai, 1997) and public areas, including sand of parks (Takai *et al*., 1996; Fernandes *et al*., 2011). Furthermore, this microorganism has emerged as a causative agent of opportunistic infections, especially in immunocompromised humans (Arlotti *et al*., 1996). In these patients the clinical manifestations are very similar to pulmonary tuberculosis, with prominent fatality rates (Muscatello *et al*., 2007). Moreover, reports of rhodococcosis infection have increased in immunocompetent hosts (von Bargen & Haas, 2009).

The discovery of virulence plasmids in *R. equi* allowed its classification as virulent, intermediately virulent and avirulent. Virulent isolates have a large plasmid that encodes a cluster of genes encoding proteins associated with virulence, including the virulence-associated protein A (vapA) (Takai *et al*., 1991). Virtually, all isolates from affected foals contain the vapA (Takai *et al*., 1996). However, several factors associated to the survival and proliferation of *R. equi*, as well as the propensity to infect the foal lungs, still remain unknown (Muscatello *et al*., 2006). Since then, numerous putative virulence factors have been described in *R. equi*, including genes potentially responsible for extracellular polysaccharides synthesis (Letek *et al*., 2010). The presence of these genes is very important, since chronic infections have been associated with the

presence of bacteria surrounded by extracellular polysaccharides (EPS), constituted principally of extracellular polysaccharide material. These aggregates composed by bacteria and EPS matrix are known as biofilm and may indicate an important convergent survival strategy among the microorganisms (Donlan & Costerton, 2002).

The biofilm is the dominant form of microbial life, and it is estimated that more than 90% of bacteria live in biofilm communities (Charaklis & Marshall, 1990). Biofilm is considered a phase of bacterial development in which the bacteria change from the planktonic form to sessile life, thus altering their physiological characteristics (O'Toole *et al*., 2000). It is estimated that, in developed countries, over 60% of treated infectious conditions are caused by biofilm formation (Chen & Wen, 2011). Many of the bacterial infections of veterinary importance are caused by biofilm-producers pathogens (Olson *et al*., 2002).

The presence of bacteria in biofilms is closely related to antimicrobial resistance and chronicity of certain microbial infections (Cucarella et al., 2004). The biofilm-forming bacteria can tolerate antimicrobial agent concentrations up to 1,000 times more than the same bacteria species in their planktonic form (Costerton *et al*., 1999; Mah & O'Toole, 2001; Jefferson, 2004). Furthermore, genetic elements related to the production of biofilms were also directly associated to multiple antimicrobial resistance (Cucarella *et al*., 2004). Diseases associate with biofilms require novel methods for their prevention, diagnosis and treatment; however, biofilm formation by bacterial pathogens of veterinary importance has received relatively little attention (Jacques *et al*., 2010). In this respect, to our knowledge, there are no studies about the biofilm formation by *R. equi* isolates from equine and environmental sources.

The goal of this study was to evaluate the biofilm formation by equine *R. equi* isolates from clinical and fecal samples using two different phenotypic methods. We also aimed correlate these methods in order to propose a useful methodological approach for screening of biofilm formation

in *R. equi*. Additionally, it was also investigated the activity of three antimicrobial commonly used in the treatment of equine rhodococcosis against planktonic and established biofilms *R. equi* cells.

METHODS

***Rhodococcus equi* isolates.** A total of 113 equine *R. equi* isolates from clinical (*n*=41) and fecal (*n*=72) samples were used in this study. The clinical samples were recovered from horse *post-mortem* pulmonary lesions subsequent to antimicrobial treatment, and the fecal samples from healthy mares. These samples were obtained from ten horse-breeding farms in the south of Brazil from 1991 to 2009 year. All the samples were characterized as *R. equi* by morpho-dyeing and biochemical testing according to Quinn *et al*., 1994 and the identification was confirmed genotypically by Monego *et al*. 2009. The *R. equi* isolates remained lyophilized and stored at -20°C until the tests be performed.

Biofilm-culturing (BC) assay. This method was chosen because is considered the gold-standard method for biofilm detection (Mathur *et al*., 2006). The quantitative determination of biofilm formation was performed by the spectrophotometric method, which measures the total biofilm biomass, including bacterial cells and EPS matrix. This assay was performed as described previously (Merino *et al*., 2009) with minor modifications. Briefly, 5 µl (≈ 10^8 colony forming units (CFU)/ml) of a culture of *R. equi* grown overnight in tryptone soya broth (TSB) medium (Himedia® Laboratories) at 37°C was inoculated into the 96-wells microtiter plates (Nunclon® Delta) containing 195 µl of TSB. After 24 h of incubation at 37°C in aerobic conditions, the microtiter plates were washed three times with 200 µl of sterile water, dried in an inverted position, and stained with 100 µl of 0.25% crystal violet for 2 to 3 min at room temperature. Then, the microtiter plates were again rinsed three times with sterile water and dried. Later, the

dye was dissolved in 200 µl of ethanol-acetone (80:20), and the absorbance was measured in ELISA microtiter-plate reader (SpectraMax®, Molecular Devices) at 570 nm-wavelength. All assays were performed in triplicate and repeated three times. Uninoculated TSB medium was used as negative control in all tests. In order to ensure the quality of the tests was also employed a reference strain of *Staphylococcus aureus* ATCC 25923 as positive control to biofilm formation (Marques *et al*., 2007). Absorbance values greater than the negative control were considered positive. It was calculated the arithmetic mean of the triplicates.

Biofilm-culturing with additional glucose (BCG) assay. All *R. equi* isolates was also analyzed by a BC assay using an additional source of energy. This procedure is similar the methodology described above, however with TSB medium supplemented with additional 0.25% glucose.

Epifluorescence microscopy (EM) assay. *R. equi* isolates were cultured in TSB medium at 37 °C during 24 h in aerobic conditions. An inoculum (200 µl) corresponding to $\approx 10^8$ CFU/mL of each *R. equi* overnight cultures grown under static conditions were distributed in a sterile petri plates (50 mm x 10 mm) containing a sterile coverslip (18 mm x 18 mm) and 3.0 ml of TSB. All the plates were incubated under the same conditions as described above. Following, the coverslip were then heat fixed and stained with 10 µl 4,6-diamino-2-phenylindole (DAPI; Sigma®) (2mg/mL) and attached to slides. The biofilm formation on coverslips was observed with an epifluorescence microscope at 100X lens 05 filter (395 nm of absorption and 440 nm of emission). Biofilm-forming bacteria were observed in conjunction with the EPS matrix. Bacteria were considered biofilm-negative when presented no EPS matrix around the stained cells. This technique was adapted from Feazel *et al*. (2009).

Planktonic antimicrobial susceptibility testing. *R. equi* isolates (*n*=8) positive in all biofilm formation tests described above were selected to carry out the antimicrobial susceptibility tests. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were performed in Müeller-Hinton broth (MHB) medium (Himedia® Laboratories) using the microdilution method in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI, 1999; CLSI, 2006). All microorganisms were cultivated in MHB for 24 h at 37 °C. For each microorganism, an inoculum suspension was prepared in 0.9% saline, adjusted to the range of 0.5 MacFarland's scale, and absorbance readings were performed in a spectrophotometer at 600 nm-wavelength. These suspensions were diluted in MHB to approximately $1x10^5$ CFU/ml. The antimicrobials tested were azithromycin, clarithromycin and erythromycin, commonly used in the treatment of equine rhodococcosis. The antibiotic concentrations tested were: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/ml. The reference strain *Staphylococcus aureus* ATCC 29213 were used for assay validation. *R. equi* ATCC 33701, known to be susceptible to azithromycin, clarithromycin and erythromycin, was also used as a control isolate (Giguère *et al*., 2010). The minimum inhibitory concentrations (MICs) were defined as the lowest concentrations of the antimicrobials that inhibited the visible growth of the *R. equi* isolates after overnight incubation, and the minimum bactericidal concentrations (MBCs) were defined as the lowest concentration of antimicrobial that prevented the growth of the *R. equi* isolates subculture on to antibiotic-free Müeller-Hinton agar (MHA) medium (Himedia® Laboratories). *R. equi* isolates were categorized as susceptible (≤ 2 µg/ml – azithromycin and clarithromycin, and ≤ 0.5 µg/ml – erythromycin) or resistant (≥ 8 µg/ml – azithromycin, clarithromycin and erythromycin) in accordance with CLSI (2008 and 2009). Additionally, isolates with MIC values between the aforementioned concentrations were categorized as intermediate susceptibility.

Biofilm antimicrobial susceptibility testing. This test was conducted in order to assess the effect of the same antimicrobials used in the test above described against established biofilms *R. equi*. The isolates analyzed were the same evaluated in the planktonic antimicrobial susceptibility test. This technique was adapted from Moreira *et al*., 2006 with modifications. Briefly, overnight bacterial cultures grown under static conditions were inoculated into TSB medium in a 1:100 dilution in 24-well cell culture plates (Falcon). These plates were incubated at 37°C during 72 h. After, all wells were rinsed three times with phosphate-buffered saline (PBS), without disturbing the adherent film; and 1 ml of the antimicrobials (8 µg/ml) diluted TSB medium and only TSB medium (control) was added on the well. These plates were incubated at 37°C for 24 h. The solutions were discarded and all wells were again rinsed three times with PBS and the biofilms were disrupted with a solution of Triton X-100 1% in PBS for 20 min. Serial dilutions were performed and plated on tryptone soya agar (TSA) medium (Himedia® Laboratories) for CFU counts. This experiment was performed in triplicate.

Statistical analysis. The Kruskal-Wallis test was employed to calculate the difference of the frequency in the biofilm formation among the methods. The correlation between the assays was performed by Spearman correlation analysis. These data were analyzed by SAS statistical software (SAS, 2001). The difference in the CFU counts among the groups was compared by one-way Analysis of Variance (ANOVA) followed by the Tukey's test. The minimum significance level considered was $P < 0.05$.

RESULTS

BC assays

The biofilm formation was observed in 46% (52/113) and 55.7% (63/113) of *R. equi* isolates in the BC and BCG, respectively. In the BC test it was verified 46.3% (19/41) and 45.8% (33/72) of biofilm-positive clinical and fecal *R. equi* isolates, respectively. When used the TSB medium supplemented with glucose, 41.5% (17/41) and 63.9% (46/72) were biofilm-positive in clinical and fecal *R. equi* isolates, respectively. Interestingly, eight clinical isolates recovered as biofilm-positive by BC became biofilm-negative in BCG, and six isolates recovered as biofilm-negative by BC had biofilm-positive phenotype in BCG. On other hand, in fecal *R. equi* isolates, 18 strains biofilm-negative in BC were observed as biofilm-positive in BCG. All fecal isolates biofilm-positive in BC remain biofilm-positive in BCG.

Epifluorescence microscopy assay

In this method, biofilm-producers *R. equi* were verified in 54.9% (62/113) of *R. equi* isolates. The biofilm-formation was observed in 39% (16/41) and 63.9% (46/72) of clinical and fecal isolates, respectively. Biofilm-positive *R. equi* colonies are demonstrated in the Fig. 1a and biofilm-negative *R. equi* colonies in the Fig. 1b.

Comparing between biofilm-culturing and epifluorescence microscopy assays

The biofilm-positive *R. equi* isolates in at least one tests performed were considered biofilm-producer. Therefore, the biofilm-formation was verified in 74.3% (84/113) of *R. equi* isolates analyzed and in 63.4% (26/41) and 80.5% (58/72) of clinical and fecal isolates, respectively.

In this study no significant difference was observed in the frequency of biofilm-formation by clinical *R. equi* isolates among the assays performed (Fig. 2a). However, it was observed an increasing in the frequency of biofilm-formation in BCG and EM assay when compared with BC

in the fecal *R. equi* isolates. Finally, the BCG and EM had the same sensibility to detection of biofilm formation in fecal isolates (Fig. 2b).

In the Spearman correlation analysis all the techniques had correlation in clinical *R. equi* isolates. Screening on BCG displayed a correlation index of 0.56 ($P = 0.0001$) with EM assay in these isolates. On the other hand, in the fecal *R. equi* isolates no correlation was observed between the BC assay with and without glucose. However, the BCG and EM assays had a strong correlation index of 0.52 ($P < 0.0001$).

Antimicrobial susceptibility testing of planktonic *R. equi* suspensions

We studied the in vitro susceptibilities of eight planktonic suspensions of *R. equi* (four from clinical samples and four from fecal samples) to antimicrobial agents of the macrolide group (azithromycin, clarithromycin and erythromycin). All isolates analyzed were susceptible to azithromycin and clarithromycin. Four isolates were classified as intermediate susceptibility and four as susceptible to erythromycin. The results of MIC and MBC are shown in Table 1.

Antimicrobial susceptibility testing of established biofilms *R. equi*

We used the antimicrobials at concentrations at least 8 times their MICs to evaluate their effects on established biofilms. The results of CFU counts (Table 2) revealed that all antimicrobial agents were able to reduce significantly the CFU of established biofilms *R. equi* cells; however, no significant difference was observed among the antimicrobial groups and between the sources (clinical and fecal) of the samples (Fig. 3). The reduction of CFU was 27.81 to 33.87%, 32.91 to 41.44% and 28.22 to 37.55% by azithromycin, clarithromycin and erythromycin, respectively (Table 2).

DISCUSSION

Biofilm formation has been considered a new life status for several microorganisms (Lemon *et al*., 2008, Feazel *et al*., 2009). However, there are few approaches about biofilm production involving veterinary microbial pathogens (Jacques *et al*., 2010). In this current study, 74.3% (84/113) of the investigated *R. equi* isolates were biofilm-positive in at least one of methodology employed. The fact of some *R. equi* isolates to appear as biofilm-positive in a determined approach and negative in another can be attributed to individual variability of the microorganisms (Stepanovic *et al*., 2000), especially at respect the source of the samples. According Knobloch *et al*., 2002, the influence of the used media and sugar supplementation in biofilm formation differed between clinical and commensal *Staphylococcus aureus* isolates. Several environmental conditions influence the biofilm formation, such as nutritional requirements and metabolic pathways (e.g. concentration glucose, iron and phosphate) (Jacques *et al*., 2010). Standardized protocol has not been established for assessment of biofilm formation by different microorganisms (Stepanovic *et al*., 2007). Different methods have been developed to study the biofilm formation, including: tube test, microtiter plate test, microscopy, Congo red agar plate test, and others (Harraghy *et al*., 2006). The microtiter plate assay remains among the most commonly assays used for investigation of biofilms, and a number of modifications have been developed in order to improve the studies on biofilms involving diverse microorganisms (Stepanovic *et al*., 2007).

In the BC assay with addition of 0.25% glucose, significantly more number of fecal *R. equi* isolates displayed a biofilm-positive phenotype (Fig. 2b), indicating a strong dependence between biofilm formation and growth environmental conditions in these isolates. Similarly, in tissue culture plate assay with TSB medium supplemented with 0.25 to 4% glucose was observed an increased in the biofilm formation by staphylococci (Knobloch *et al*., 2002). In contrast, between

BC and BCG assays no statistical significant difference was detected in regarding to biofilm formation in clinical *R. equi* isolates (Fig. 2a). In these isolates, the presence of glucose appears no influence the biofilm formation. Thus, we believe that different regulatory mechanisms could be active in expression of biofilm between pathogenic and environmental *R. equi*.

In the current study, 80.5% (58/72) of fecal *R. equi* isolates were biofilm-producers. This great number of fecal *R. equi* isolates biofilm-producers may be associated with the protection ensured by the EPS matrix, an arrangement used to survive in the stress conditions which these bacteria are submitted in the environment (Heipieper *et al*., 1991). As well documented, the biofilm formation provides protection to microorganism from a wide range of environmental adversities, including exposition at UV radiation (Espeland *et al*., 2001) and metal toxicity (Teitzel & Parsek, 2003). Moreover, the characteristics of biofilms growth visualized in different environments are noticeably similar, suggesting that it is an important convergent survival strategy among the microorganisms (Hall-Stoodley *et al*., 2004). Exposure to *R. equi* is widespread in environment, a study demonstrated 76% of mares shed virulent *R. equi* in their feces during peri-partum (Buntain *et al*., 2010). Additionally, *R. equi* was isolated from feces of 3-week-old (93%) and 4-week-old (96%) foals (Chaffin *et al*., 2008). According to Kaplan, 2010 microorganisms detached from biofilm have an important role in bacterial dissemination of environmental reservoirs to human or animal hosts, as well as the microorganism transmission among hosts. In this respect, our findings allow infer that a greater number of pneumonia cases in foals may be occurring due infection by fecal *R. equi* biofilm-producers and no only by pathogenic samples.

It was verified 63.4% (26/41) of clinical *R. equi* isolates as biofilm-producers. Commonly, biofilms are associated to infections with prolonged treatment protocols (Kulka *et al*., 2012), as well as the recurrence or persistence of infections (Hall-Stoodley *et al*., 2004). *R. equi* infections usually have a long-term antimicrobial therapy, commonly 4–9 week of treatment (Hillidge,

1987), besides these infectious became chronic (Prescott, 2004). A recent study described the first report of three human *R. equi* isolates biofilm-producers in central venous catheter using a scanning electron microscopy (Akhrass *et al*., 2012). This observation confirms earlier studies about the association between biofilm and chronic infections.

Martın-de-Hijas *et al*., 2009 demonstrated biofilm assortment as an important issue for developing infections of mycobacteria strains. In biofilm-forming *Mycobacterium tuberculosis* was observed that the EPS matrix sustains drug-tolerant sub-populations, which persist even though to high exposure to rifampicin (Jaques *et al*., 2010). Additionally, clarithromycin has no activity against pre-established biofilm *M. tuberculosis* strains (Carter *et al*., 2004). In general, the MIC of planktonic bacteria has no correlation with the concentration required to eradicate biofilms (Jacques *et al*., 2010). In a prior study was demonstrated that the range of 4 x MIC could eradicate the preformed biofilms of Gram-positive bacteria (Raja *et al*., 2011), what explains the use of 8 µg/ml (8 times the MIC) antimicrobial concentration in our biofilm antimicrobial susceptibility test. In this study, the antimicrobials used were able to reduce the CFU in established biofilm *R. equi* and the clarithromycin showed the greater percentage of reduction (up to 40%). The reduction in the CFU observed in this study was significant when compared with the control group (Fig. 3), however did not eradicate the biofilm *R. equi* cells. These findings corroborate with Akhrass *et* al., 2012, which demonstrated that injectable antimicrobial solutions containing 1 mg/mL vancomycin had limited biofilm penetration and activity against biofilm *Rhodococcus* spp.

R. equi infects macrophages and causes lung infections similar to pulmonary tuberculosis (Muscatello *et al*., 2007); likewise the antimicrobial association of rifampicin and clarithromycin has been preconized for treatment of rhodococcosis (Muscatelo, 2012). We believed that the extensive period of antimicrobial therapy employed to treat equine rhodococcosis can induce

and/or increase the biofilm formation, resulting in therapeutic failure observed in many studies about *R. equi* antimicrobial resistance.

The epidemiology and pathogenesis of *R. equi* infection may be affected by the kind of life in biofilm. O'gara & Humphreys, 2001 consider the extracellular polysaccharide adhesin, required for biofilm formation, a key virulence determinant in *Staphylococcus epidermidis*; thus we emphasize the importance of biofilm formation as epidemiological marker in *R. equi*. At respect of pathogenesis, the sessile cells from biofilm-producers pneumococcal are more effective in inducing pneumonia than planktonic cells (Oggioni *et al*., 2006); this may be happening in foal pneumonia by *R. equi*. Likewise, microorganisms that are growing in EPS matrix are able to resist to host's defenses, since biofilms protect bacteria from the immune system (Lemon *et al*., 2008) and act as reservoirs for antibiotic resistance genes (Jacques *et al*., 2010).

We emphasized the importance of further studies, including the engineering biofilm formation and its dispersal; as well as the functions, the properties and the main constituents of the EPS matrix in biofilm *R. equi*. Since, the elucidation of the mechanism of *R. equi* biofilm formation and its structure may lead to new preventive measures, including: novel substances anti-adhesion, quorum sensing disruption and selective targeted anti-antimicrobial peptides (Chen & Wen, 2011).

In conclusion, the *R. equi* isolates analyzed are able to form biofilm in all methodological approaches tested. The biofilm formation by fecal *R. equi* isolates is more frequent than in clinical isolates. Both BCG and EM methods are adequate to screening of biofilm formation in fecal *R. equi* isolates. No significant difference among all biofilm assays was observed in clinical *R. equi* isolates, furthermore, the addition of 0.25% glucose does not appear to influence the biofilm formation by this isolates. Although the isolates analyzed show a susceptibility profile, the antimicrobials tested herein were not able to eradicate the *R. equi* biofilm in the concentration

employed. Finally, the evaluation of biofilm susceptibility *R. equi* may be a more appropriate approach to correlate the susceptibility in vitro with clinical outcome resulting from a prolongated treatment of rhodococcosis.

Abbreviations: CFU, colony forming units; CLSI, Clinical Laboratory Standards Institute; DAPI, 4,6-diamino-2-phenylindole; EPS, extracellular polysaccharides; MBC, minimum bactericidal concentration; MHA, Müeller-Hinton agar; MHB, Müeller-Hinton broth; MIC, minimum inhibitory concentration; NANAT, nalidixic acid–novobiocin–actidione (cycloheximide)–potassium tellurite; PBS, phosphate buffer solution; TSA, tryptone soya agar; TSB, tryptone soya broth.

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Table 1. In vitro susceptibilities of planktonic clinical and fecal *R. equi* isolates to azithromycin, clarithromycin and erythromycin.

Strain (n = 8)	MIC - MBC (µg/ml)		
	Azithromycin	Clarithromycin	Erythromycin
R. equi 488/00	1.0[a] - 2.0	0.25[a] - 0.25	0.5[a] - 1.0
R. equi 27/98	1.0[a] - 2.0	0.125[a] - 0.125	2.0[b] - 2.0
R. equi 353/93	0.125[a] - 0.5	0.25[a] - 0.25	0.5[a] - 1.0
R. equi 25/03	1.0[a] - 2.0	0.0625[a] - 0.0625	1.0[b] - 2.0
R. equi 490/95 DID	1.0[a] - 2.0	0.125[a] - 0.125	1.0[b] - 2.0
R. equi 490/95 INB	0.5[a] - 2.0	0.0625[a] - 0.0625	0.5[a] - 1.0
R. equi 490/95 TRA	1.0[a] - 2.0	0.0625[a] - 0.0625	2.0[b] - 2.0
R. equi 490/95 IST	0.5[a] - 2.0	0.0625[a] - 0.0625	0.5[a] - 1.0

[a] Susceptible strain; [b] Intermediate susceptibility strain; both in accordance with CLSI, 2008 and CLSI, 2009.

Table 2. Bactericidal activities of azithromycin, clarithromycin and erythromycin against established biofilm *R. equi* isolates in 24-h-old biofilms.

Strain (n = 8)	Bacterial count ($\log_{10}$ CFU/ml)[a] - % reduction			
	Biofilm control	Azithromycin	Clarithromycin	Erythromycin
R. equi 488/00	8.49 – NA	5.77 – 32.06	5.00 – 41.14	5.48 – 35.52
R. equi 27/98	8.16 – NA	5.84 – 28.48	5.48 – 32.91	5.86 – 28.22
R. equi 353/93	8.34 – NA	5.89 – 29.32	5.40 – 35.25	5.51 – 33.96
R. equi 25/03	8.26 – NA	5.83 – 29.39	5.04 – 38.97	5.85 – 29.24
R. equi 490/95 DID	8.27 – NA	5.65 – 31.68	4.85 – 41.44	5.53 – 33.19
R. equi 490/95 INB	8.07 – NA	5.83 – 27.81	5.05 – 37.45	5.74 – 28.86
R. equi 490/95 TRA	8.08 – NA	5.34 – 33.87	4.90 – 39.31	5.05 – 37.55
R. equi 490/95 IST	8.28 – NA	5.51 – 33.48	4.93 – 40.44	5.23 – 36.77

[a] The results are obtained from a poll of three wells; this poll was submitted to serial dilutions and it was plated on TSA; the cells were counted after 24 h of incubation at 37°C; NA, not applicable.

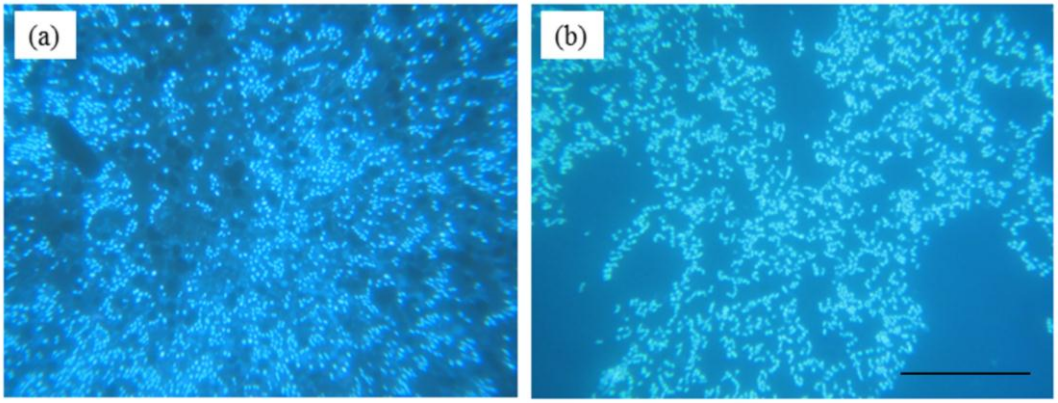

Fig. 1. Biofilm formation by *R. equi* isolates. The bacteria were grown on a glass slide in 24 h, stained with DAPI and examined with an epifluorescence microscopy (100 X). (a) Aggregate of cells of *R. equi* surrounded by EPS matrix. (b) *R. equi* cells without EPS matrix. Scale bar 20 μm.

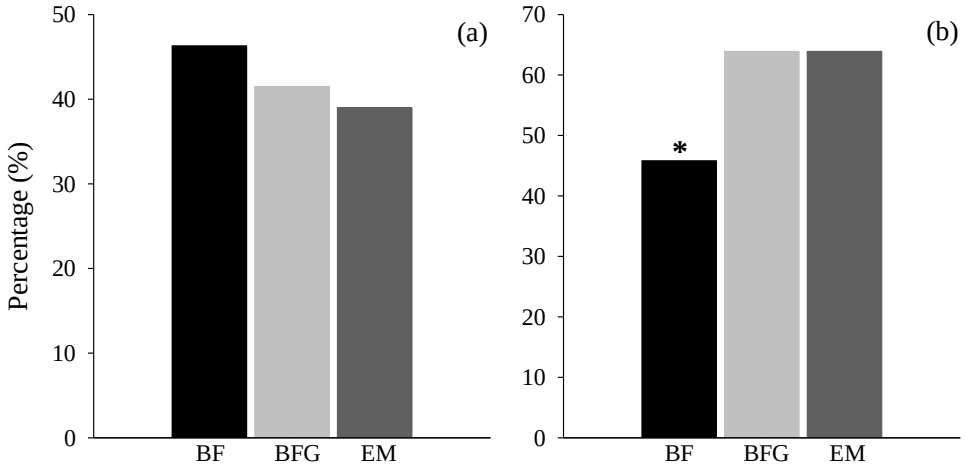

Fig. 2. Frequency of biofilm formation by *R. equi* isolates evaluated by three different methodological approaches. Legend: BF – biofilm-forming, BFG – biofilm-forming with glucose supplementation and EM - epifluorescence microscopy. (a) Clinical isolates frequency. (b) Fecal isolates frequency. (*) Indicates significant difference among the different assays by Krustal-Wallys test ($P < 0.05$).

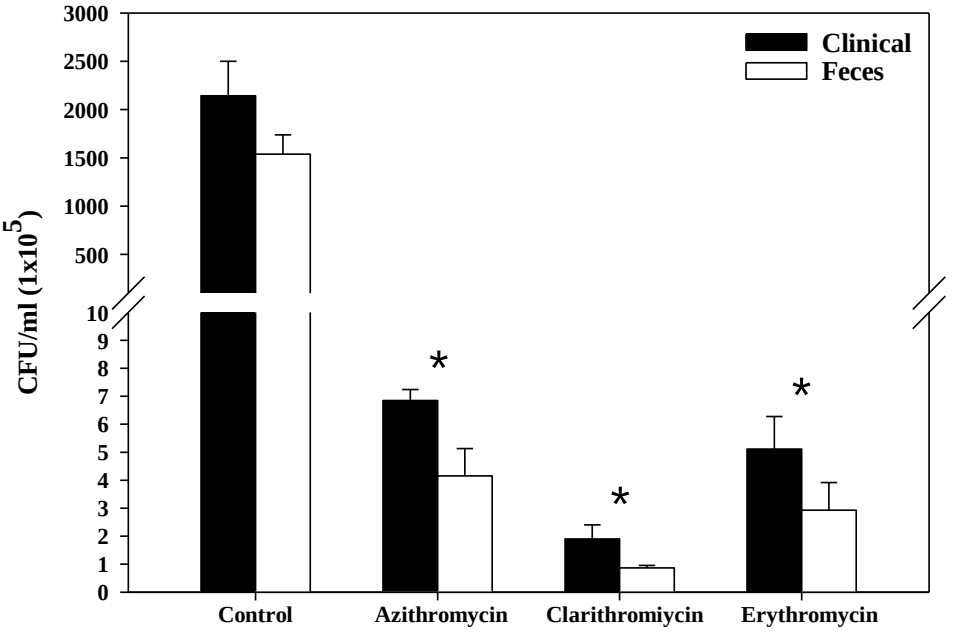

Fig. 3. CFU/ml in *R. equi* established biofilms treated with three antibiotics commonly used in the treatment of equine rhodococcosis. Clinical (n = 4) and fecal (n = 4) *R. equi* isolates. (*) Indicates statistically significant difference from control by one-way ANOVA and Turkey's test ($P < 0.05$).

3. ARTIGO II

(Artigo a ser submetido ao periódico Research in Veterinary Science)

GENOTYPIC AND PHENOTYPIC DETECTION OF EFFLUX PUMP IN *Rhodococcus equi*

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Abstract

The gene *req_39680* encodes a protein associated to a putative efflux system, important mechanism of antimicrobial resistance, was described in the reference strain *Rhodococcus equi* 103S. We verified the presence of this gene in 90 *R. equi* isolates from different sources as well as its presence was correlated with phenotypic expression of efflux mechanism and *vapA* gene occurrence. The PCR was employed for detection of the *req_39680* gene. The phenotypic expression of efflux mechanism was verified in agar containing ethidium bromide. The gene was detected in 60% of the isolates and phenotypic expression of efflux mechanism in 20%. The correlation index between the presence of *req_39680* gene and phenotypic expression of efflux mechanism was 0.66 in clinical isolates. No correlation was observed between *vapA* and *req_39680* genes. The efflux systems appear to be an emerging form of adaptation to pathogenic and saprophytic life by *R. equi* isolates from different sources.

Key words: ethidium bromide; efflux pump; multidrug resistance; *Rhodococcus equi*; *vapA*

1. Introduction

Efflux systems or efflux pumps are transporter proteins of toxic compounds classified into five families according to their primary structure and energy-coupling mechanism: i. Major Facilitator Superfamily - MFS ii. Adenosine Triphosphate-Binding Cassette - ABC, iii. Small Multidrug Resistance Family - SMR, iv. Resistance-Nodulation-Cell Division Superfamily - RND, and v. Multidrug and Toxic Compound Extrusion Family - MATE (Kumar and Schweizer, 2005). These proteins were firstly described in *Escherichia coli* (Mcmurray et al., 1980).

Although the majority of genes encoding efflux pumps can be found on plasmids, there are other efflux pump genes located on the chromosome, which provide the bacterium an intrinsic

mechanism that allows survival in a hostile environment (e.g. the presence of antibiotics) (Webber and Piddock, 2003). Then, the efflux pumps are related to acquired and intrinsic resistance to several antibiotics in different bacterial species *(*Levy, 1992; Li and Nikaido, 2004*)*. Cross-resistance was already described in efflux systems, i.e. the exposure to any one agent that belongs to the substrate profile of an efflux pump can allows its over-expression and consequent cross-resistance to different substrates (Webber and Piddock, 2003). These systems are also considered as a major mechanism of resistance among multidrug-resistant pathogenic microorganisms in veterinary medicine (Lyon and Skurray 1987; Paulsen, 2003).

Rhodococcus equi is a facultative intracellular pathogen that infects animals and immunocompromised human patients (Takai et al., 1991; Takai et al., 2000). This bacterium is the etiological agent of rhodococcosis, an important disease that typically affects foals less than six months of age, causing severe lesions including pyogranulomatous pneumonia and mesenteric lymphadenitis (Prescott, 1991). The most important virulence factor is associated with the presence of plasmids encoding genes associated with virulence proteins (Vap) (Takai et al., 1995), which has been used to classify isolates as virulent (VapA); intermediately virulent (VapB) and avirulent (without virulence plasmid) (Ribeiro et al., 2005).

Currently, the treatment of rhodococcosis usually consists of a combined application of macrolides, such as erythromycin, azithromycin or clarithromycin, and rifampicin (Muscatello, 2012). However, the resistance in *R. equi* isolates to various antimicrobials, including the macrolides group, has been reported (Takai et al., 1997; Asoh et al., 2003; Niwa et al., 2006; Buckley and Stanbridge, 2007; Venner et al., 2011). The microorganisms' resistance to macrolides has been associated to basically three events: presence of methylases, efflux pump activity and enzymatic inactivation (Sutcliffe et al., 1996).

In *R. equi* the presence of four putative multidrug efflux pumps was mentioned in the reference strain *R. equi* 103S (Letek et al., 2010). This study reported the sequence of a chromosomal gene known as *req_39680* which encodes a protein, with 308 amino acids, related to putative cation efflux system. Similar efflux mechanism was described in mycobacteria being related to the development of high level drug-resistance (Schmalstieg et al., 2012). However, studies about efflux systems activity have not been described in *R. equi*. In order to evaluate the occurrence and expression of efflux pumps in *R. equi* isolates, this study aimed to identify the presence of the *req_39680* gene, as well as to verify the phenotypic expression of efflux pump in avirulent and virulent *R. equi* isolates.

2. Material and methods

2.1. Bacterial isolates

R. equi isolates were obtained from ten horse-breeding farms, located in the Brazilian states of Rio Grande do Sul (RS) and Paraná (PR). The isolates were obtained from soil samples (n=30), feces (n=30) of healthy animals and foals clinical samples (n=30), which were recovered from post-mortem pulmonary lesions subsequent to antimicrobial treatment, from 1991 to 2012 year. The reference strains *R. equi* ATCC 33701P + (*vapA* positive), *R equi* ATCC 33701P - (*vapA* negative) and *Staphylococcus aureus* ATCC 25923 were used as control in both phenotypic and genotypic assays. *Mycobacterium goodii* SB314/96, *Nocardia* sp. SB57/2008, were used as negative control in the genotypic assays.

Among 90 bacterial isolates analyzed, 70 were identified phenotypic and genotypically previously by Monego et al. (2009), being classified as *vapA* positive *R. equi* (n=26) and *vapA* negative *R. equi* (n=44). These isolates were lyophilized and stored at -20 °C. Additionally, 20 soil samples were collected in January of 2012 in three different horse-breeding farms in RS. The

soil samples (≈10 g) were collected from mare-foal paddocks (n=20) and stored in sterile plastic bags until the bacteriology culturing. These samples were growing in nalidixic acid–novobiocin–actidione (cycloheximide)–potassium tellurite (NANAT) selective medium described by Woolcock et al. (1980). Colonies with *R. equi* profile were submitted to phenotypic identification (Quinn et al., 1994) and genotypic analysis for genus, species and *vapA* gene (Takai et al., 1995). All the *R. equi* soil isolates were classified as *vapA* negative and also were lyophilized and stored at -20 °C.

2.2. Putative cation efflux protein analysis

This analysis was performed in order to evaluate the characteristics of the protein encoded by *req_39680* gene prior to perform the genotypic test. The sequence of the protein was recovered from *R. equi* 103S (GenBank access YP_004008630.1) and submitted to analysis by the Phyre V 2.0 (Protein Homology/Analogy Recognition Engine) program (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.3. Molecular detection of the chromosomal gene req_39680 in R. equi isolates

To detect the *req_39680* gene in the *R. equi* isolates the polymerase chain reaction (PCR) method was used. Primarily, each strain was grown on Müeller Hinton agar (Himedia® Laboratories) and incubated at 37 °C for 24 h. Three to five colonies were suspended in 500 μL of Milli-Q water and subjected to DNA extraction by cethyltrimethylammonium bromide (CTAB) protocol (Sambrook and Russell, 2001). The extracted DNA was measured using the Picopet01 DNA Calculator (Cambridge, England).The PCR primers employed were: forward RE1 (5'-CCGCGATCCCTCGACACACG-3') and reverse RE2 (5'-CCCACCCGCATCCGCAAGAT-3'). Both primers were designed using Primer-BLAST

program (http://www.ncbi. nlm.nih.gov/tools/primer-blast/) based on DNA sequence of the reference strain *R. equi* 103S, deposited in GenBank (NC_014659.1) (http://www.ncbi.nlm.nih.gov) in order to amplify the complete gene. The PCR was carried out in a total volume of 25 μL containing: 10 μmoles of each primer, 200 μM of deoxynucleotides (dNTPs, Invitrogen®), 1 U of DNA polymerase (GoTaq, Invitrogen®), 1X of ®the 5X enzyme buffer, and 60 ng of DNA sample. The amplifications were performed using PTC-100 Programmable Thermal Controller (MJ Research) with the following cycling profile: initial denaturation at 94°C/1min, 35 cycles of 94 °C/30 s, 63 °C/30 s and 72 °C/1 min 30 s, and a final extension at 72 °C/5 min. PCR products (10 μL) were run in 1% agarose gel, stained with ethidium bromide, visualized under UV light and photo documented by L-PIX ST (Loccus® Brazil). All PCR products were purified, in quadruplicate, with the PureLink PCR Purification Kit (Invitrogen®) and sent for DNA sequencing with the same primers for *req_39680* gene. DNA sequencing was performed in an automated sequencer ABI-PRISM 3100 Genetic Analyzer (ACT Gene Molecular Analysis Ltd., Biotechnology Center/UFRGS, Porto Alegre, RS). DNA consensus sequences were generated by Gap program of the Staden package 4 software (Staden et al., 2000) and analyzed by Basic Local Alignment Search Tool (BLAST) (NCBI/DNASIS software version 2.5, Software Engineering Co. Ltd., SanBruno, California, US).

2.4. Phenotypic expression of efflux mechanism in agar containing ethidium bromide (EtBr)

This assay was based on the use of a fluorescent cationic dye (EtBr) to visualize the efflux mechanism in bacterial cells (adapted from Martins et al. (2006). Six *R. equi* isolates from clinical (n=2), feces (n=2) and soil (n=2) samples were tested with different concentrations of EtBr (0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 μg/mL) in order to determine the toxicity level of this dye for cells. Each group had an isolate presenting *req_39680* gene. The reference strain *S.*

aureus ATCC 25923 was used as positive control according Couto et al. (2008). All microorganisms were cultivated in tryptic soy agar (TSA) medium (Himedia® Laboratories) for 24 h at 37 °C. A single colony of each bacterium was transferred to tryptic soy broth (TSB) medium (Himedia® Laboratories) and maintained at 37 °C overnight. For each microorganism, an inoculum suspension was prepared in 0.9% saline, adjusted to the range of 0.5 MacFarland's scale, and absorbance readings were performed in a spectrophotometer at 600 nm-wavelength. Subsequently, dilutions in TSB were carried out to obtain an inoculum containing approximately 1,000 CFU/mL. The inoculum was applied with swab on plates containing EtBr incorporated into the TSA and incubated at 37 °C for 48 h under aerobic conditions. Later, these plates were visualized on UV light. Both viability of cells and capacity of detection of efflux systems at the different concentrations were observed. The concentration of EtBr at 0.2 µg/mL was selected to be used in all isolates, since provided the adequate physiological conditions to development of the *R. equi* isolates. All tests were performed in duplicate. After the visualization of the results on UV light the plates were photo documented by PIX-L ST (Loccus® Brazil). Bacteria were considered positive to efflux system when the fluorescent colonies were not observed under UV light and negative when the colonies showed fluorescence.

2.5. Statistical method

The data were analyzed by SAS statistical software (SAS, 2001). The nonparametric Chi-square test was used to calculate the difference in the *req_39680* gene frequency among *R. equi* isolates from different sources. Likewise, it was used the Spearman correlation analysis in order to evaluate the correlation between the presence of this gene with the phenotypic expression of efflux mechanism, as well as the occurrence of *vapA* gene. The minimum significance level considered was $P < 0.05$.

3. Results

3.1. Putative cation efflux protein of R. equi

The protein encoded by *req_39680* gene demonstrated 100% confidence and 96% coverage with MATE family transporter protein. This protein was classified as multi antimicrobial extrusion protein and characterized as cation-bound multidrug and toxin compound extrusion protein when analyzed by Phyre V 2.0.

3.2. The req_39680 gene in R. equi isolates

The *req_39680* gene was detected in 60% (54/90) of *R. equi* isolates tested (Table 1), as well as in *R. equi* ATCC 33701. All PCR- products amplified a specific DNA fragment of approximately 840 bp (Figure 1). The identity of *R. equi* PCR products was confirmed by DNA sequence consensus analyses, which showed 100% of similarity with the reference strain *R. equi* 103S (GenBank access NC_014659.1). A DNA sequence from a Brazilian *R. equi* isolate (SB 54/97) was deposited in GenBank (access JX_512957).

3.3. Phenotypic expression of efflux mechanism in R. equi isolates

The efflux mechanism by extruding of EtBr (Figure 2) was detected in 20% (18/90) of *R. equi* isolates tested, including: 61.1% (11/18) clinical, 22.2% (4/18) soil and 16.7 % (3/18) feces isolates (Table 1). Additionally, the *req_39680* gene was detected in all positive samples for efflux mechanism by extruding of EtBr. However, 66.6% (36/54) of *req_39680* gene positive *R. equi* isolates did not show phenotypic expression of efflux mechanism.

3.4. Frequency of req_39680 gene and correlation analysis

The *req_39680* gene was detected in *R. equi* isolates from clinical (66.7%), soil (66.7%) and feces (46.7%) samples. No difference was observed in the frequency of *req_39680* gene among *R. equi* isolates from different sources.

The presence of the *req_39680* gene and phenotypic expression of efflux mechanism had correlation index of 0.66 ($P < 0.0001$) for clinical isolates. No correlation was observed in *R. equi* isolates from feces and soil. Moreover, no correlation was also observed between the presence of *vapA* and *req_39680* genes.

4. Discussion

Efflux systems associated to antimicrobial multiresistance profile have been described in bacteria determining important infections to humans and animals, including *Campylobacter jejuni*, *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Salmonella typhimurium*, and *Staphylococcus aureus* (Webber and Piddock, 2003). However, this resistance mechanism was not reported in *R. equi*.

Although Letek et al. (2010) have described four putative efflux mechanisms in the reference strain *R. equi* 103S, no exist additional information about the genes related with these systems. Thus, due to the data observed by analysis of the protein encoded by *req_39680* gene, this gene was selected to be more researched in *R. equi*. This study demonstrated the presence of the *req_39680* gene in 60% of *R. equi* isolates tested by PCR. This finding is very relevant since the protein encoded by *req_39680* gene has 100% confidence with MATE family transporter protein, categorized among the multidrug efflux transporter families present in various pathogenic microorganisms (Kuroda and Tsuchiya, 2009).

In this work, the *req_39680* gene was detected in both *R. equi* ATCC 33701 strains analyzed. This result corroborates with the described by Rahman et al. (2003), which demonstrated the *R. equi* ATCC 33701 has 12 putative genes encoding efflux proteins, as well as 25 proteins of the ABC family, some of which are related to drug resistance. In addition, both reference strains, *R. equi* ATCC 33701 and 103S, are genetically similar; they were isolated from clinical cases of pneumonia in foals (Rahman et al., 2005) and also their virulence plasmid sequences are virtually identical (Takai et al., 2000).

We suggest that the *req_39680* gene should be equally present in clinical, feces and soil *R. equi* isolates since no difference was detected in the frequency of *req_39680* gene among *R. equi* isolates from different sources. Likewise, Letek et al. (2010) observed some antimicrobial resistance genes associated to efflux systems in clinical isolates of *R. equi* as well as other environmental rhodococci. The capacity of environmental microorganisms in ensuring an effective survival and colonization in their ecological niche due to the efflux of toxic substances was previously described by Piddock (2006). Martinez (2009) reported that even in places where there is no direct exposure to antimicrobial compounds some environmental microorganisms have been adapted and developed mechanisms to defend themselves from harmful compounds.

Other members of the genus *Rhodococcus*, such as *Rhodococcus erythropolis* and *Rhodococcus fascians*, assure their survival in the environment through the efflux pumps (Desomer et al., 1992; Nagy et al., 1997*)*. In our study, a great number of *R. equi* isolates of soil and feces samples were *req_39680*-positive. Then, these data allow us to infer that both soil and feces isolates presenting *req_39680* gene may be highly adapted to survival in the environment. This fact may have influence in the epidemiology of the rhodococcosis since *R. equi* is predominantly telluric and the soil is the major source of infection (Prescott, 2004). According Martinez (2009) drug resistance determinants present in soil organisms typically may have an

important impact on the clinical management of microbial infections. This was observed in the strain *R. equi* 103S by genomic analysis (Letek et al., 2010). Finally, the data reflect that the gene is present in a comparable proportion of isolates recovered from all three sources, suggesting that the presence of the gene is not an adaptation to the host but may be just as relevant to its saprophytic status, given that aerosols from soil or fecal derived strains are the most likely source of rhodoccocal pneumonia (Muscatello et al., 2006).

The treatment failures observed in infections by *R. equi* may be associated to the extrusion of chemical compounds (e.g. antimicrobials) once resistance mechanisms to macrolides also include two families of efflux pumps (Roberts et al., 1999). Additionally, studies involving different microorganisms revealed that efflux pumps were usually capable of extruding multiple compounds, including substances structurally unrelated (Paulsen, 2003), increasing the chances of failure in infection control. Considering the data obtained in this work, where 66.7% clinical isolates of *R. equi* were *req_39680*-positive, we underscored the necessity of more researches about efflux mechanisms in *R. equi*.

The assessment of phenotypic expression of efflux mechanism by methodologies using EtBr as substrate is widely employed in phenotypic studies to verify efflux pumps (Li and Nikaido, 2004*, 27)*. However, these methodologies are based on qualitative results and have also show limitation due to physiological characteristics of each microorganism (Martins et al., 2006). In this study, in order to reduce misleading results, the phenotypic expression of efflux mechanism assay was firstly standardized with different EtBr concentrations. After, all *R. equi* isolates were tested at 0.2 µg/mL of EtBr, since this concentration no affect the best growth of the strains. We suggest that this methodological approach could be a useful to further screening assays of efflux systems in *R. equi*, furthermore, this is an instrument-free method for the demonstration of efflux pump activity of bacteria (Martins et al., 2006).

Although 60% of *req_39680* gene-positive *R. equi* isolates did not show phenotypic expression of efflux mechanism, this mechanism may be expressed by these isolates in other circumstances to be evaluated. In this respect, Viveiros et al. (2005) demonstrated that an *E. coli* strain sensitive to tetracycline became resistant due to the efflux mechanism induced after slow and gradual exposure to the antibiotic and this expression was reverted by serial transfer to drug-free medium or by exposure to inhibitors of efflux pumps.

In contrast, a high correlation index (0.66; $P < 0.0001$) was verified between the presence of the *req_39680* gene and phenotypic expression of efflux mechanism in clinical isolates. Interestingly, the majority of these isolates were resistant to vancomycin (data not shown), a cationic drug used for treating human rhodococcosis (Bowersock et al., 2000). The data observed in this study allow to presume that *R. equi* clinical isolates have potential to extrude cationic compounds, such as the vancomycin, by efflux pumps. Furthermore, the fact of these clinical samples have been recovered from foals post-mortem pulmonary lesions, subsequent to antimicrobial treatment against *R. equi*, may be driving an expression of the gene in the host.

According to Letek et al. (2010) the correlation between the presence of virulence and antimicrobial resistance genes in *R. equi* is not well documented. As stated by these same authors, it is possible the occurrence of a direct regulatory interaction or only a random effect between these genes. In this study, no correlation was observed between the presence of both *vapA* and *req_39680* genes in *R. equi* isolates. This finding may be better explained by the location of *req_39680* gene, since it has a chromosomal *locus* and the *vapA* is a plasmidial gene.

Several studies concerning efflux systems have analyzed the influence of these systems on the antimicrobial resistance and the pathogenesis of infectious agents. All efflux pumps families, except the ABC family, use an electrochemical gradient of cations through the membrane to transport toxic compounds as EtBr, which is pumped out via several MATE transporters (Kuroda

and Tsuchiya, 2009). Based on the results observed in our research we highlighted the importance of further studies in order to evaluate the implications of efflux systems in the relationship between *R. equi* and hosts, as well as *R. equi* and environment. Other studies also might include the antimicrobial susceptibility patterns or adaptation to saprophytic status by *R. equi*.

In conclusion, the *req_39680* gene is equally distributed among the *R. equi* isolates from different sources. It was observed a high correlation index between phenotypic expression of efflux mechanism and presence of *req_39680* gene in clinical samples. Thus, there is a potential possibility of efflux systems to be an emerging form of adaptation to pathogenic and saprophytic life by *R. equi* isolates; however, additional studies are required to confirm this hypothesis.

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LT Gressler is a PhD student in the Bacteriology Laboratory at Universidade Federal de Santa Maria (Federal University of Santa Maria) (UFSM), Brazil. She has been working in resistance mechanisms of *Rhodococcus equi* and this manuscript is a part of her master's thesis. Her research interests include the epidemiology and pathogenesis of *R. equi*.

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Table 1. Molecular detection of the gene *req_39680* and phenotypic expression of efflux mechanism in agar containing ethidium bromide (EtBr) in clinical, feces and soil *R. equi* isolates (*n*=90).

Source	Positive/total of *R. equi* isolates	
	req_39680 gene PCR	Phenotypic expression of efflux mechanism
Clinical	20/30*	11/30*
Feces	14/30	03/30
Soil	20/30	04/30
Total	54/90	18/90

*A high correlation index (0.66; P < 0.0001) was verified between the presence of the *req_39680* gene and phenotypic expression of efflux mechanism by Spearman correlation analysis.

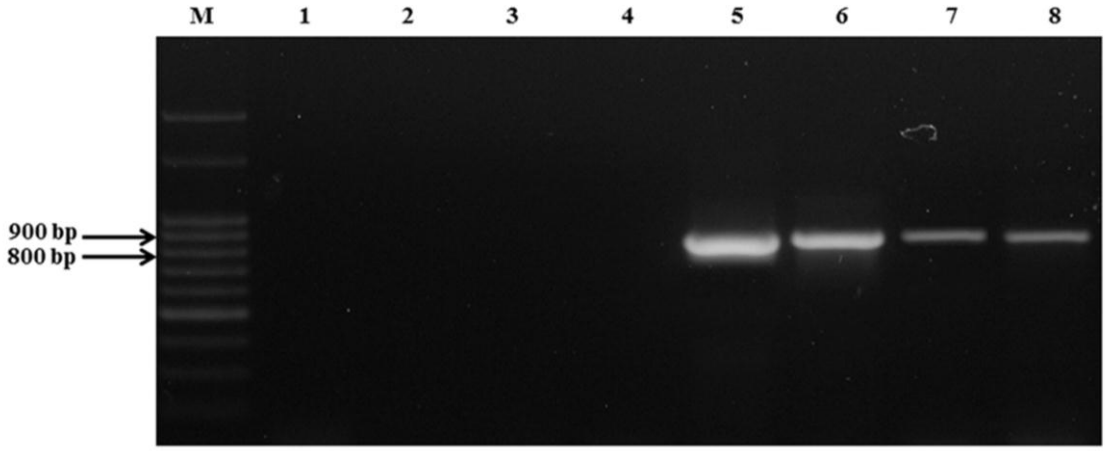

Figure 1. PCR amplification patterns of *req_39680* gene in *R. equi* isolates. Amplicons were generated from a single PCR assay with *R. equi req_39680* gene-specific primers (approx. 840 bp). Line 1: Molecular weight marker, 100 bp-DNA ladder (Ludwig Biotech®, Brazil); Lines 2-4: Negative controls (Ultra-pure water, *Mycobacterium goodii* SB314/962, *Nocardia* sp., respectively); Line 5: reference strain *R. equi* ATCC 33701 (positive control); Lines 6-8: Samples from clinical, feces and soil isolates (*R. equi* SB54/97, SB490/95, 20/12, respectively).

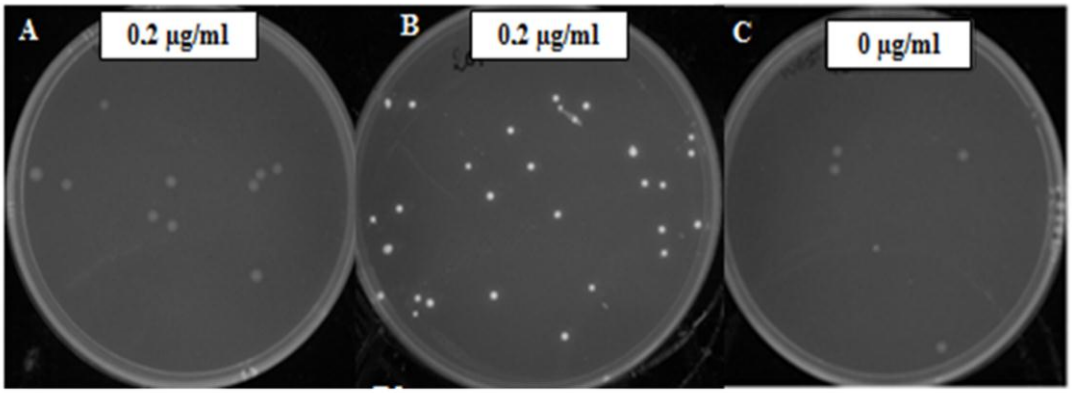

Figure 2. Phenotypic expression of efflux mechanism by *R. equi* in agar containing EtBr [0.2 µg/mL]. A: Positive-efflux mechanism in *R. equi* SB54/97 isolate, B: Negative-efflux mechanism in *R. equi* SB27/98 isolate and C: Agar without EtBr. Images captured by L-PIX ST (Loccus®, Brazil).

4. DISCUSSÃO

Diversos aspectos relacionados à patogenicidade de *R. equi* foram estudados, incluindo a aderência em células do hospedeiro, a cinética da sobrevivência no interior de macrófagos, e a resposta imune específica do hospedeiro necessária à proteção contra a infecção (HONDALUS, 1997). No entanto, o grande avanço no entendimento da patogênese ocorreu com o descobrimento de plasmídeos de alto peso molecular, altamente correlacionados à virulência em animais (TAKAI et al., 1991). Outros mecanismos de resistência, como por exemplo, formação de biofilme e bombas de efluxo permanecem praticamente desconhecidos em *R. equi*, especialmente entre os isolados oriundos de amostras de equinos e do ambiente.

Recentemente, o primeiro relato de formação de biofilme por *R. equi* foi publicado; contudo, apenas três isolados de origem humana foram analisados (AKHRASS et al., 2012). Nos últimos anos, estudos escassos sobre biofilmes em patógenos de relevância médica veterinária foram desenvolvidos (JACQUES et al., 2010). Todavia, este é um tema extensamente estudado em bactérias envolvidas em infecções humanas (LEMON et al., 2008). Em relação às bombas de efluxo, alguns trabalhos mencionam a existência de possíveis mecanismos de efluxo ou proteínas relacionadas à extrusão de compostos tóxicos (RAHMAN et al., 2003; LETEK, et al., 2010) envolvendo este micro-organismo. No entanto, estudos avaliando a presença de genes específicos de efluxo e de expressão deste mecanismo em isolados de *R. equi* ainda haviam sido desenvolvidos.

Em ambientes naturais, as células bacterianas são mais frequentemente encontradas em estreita associação com superfícies e interfaces, sob a forma de biofilmes (BRANDA et al., 2005). De acordo com DAVEY & O'TOOLE (2000) os biofilmes oferecerem aos micro-organismos saprófitos vários benefícios incluindo a proteção contra compostos tóxicos. Além disso, em muitos casos, a atividade metabólica das populações microbianas em biofilmes é maior do que aquelas cujos micro-organismos estão em vida livre (ORR et al., 2004). A pesar de *R. equi* ser patogênico, este micro-organismo também é comumente encontrado no ambiente (HUGHES & SULAIMAN, 1987). MIRANDA-CASOLUENGO et al. (2008) revelaram que genes presentes neste micro-organismo são responsáveis por sistemas relevantes de aquisição de ferro em solos pobres, demostrando sua capacidade de sobrevivência mesmo em condições adversas. Deste

modo, *R. equi* pode sobreviver em condições ambientais extremas, tais como baixo pH e estresse oxidativo (BENOIT et al., 2000). Sendo assim, uma das principais justificativas para formação de biofilmes por *R. equi* pode advir de sua característica predominantemente telúrica (PRESCOTT & HOFFMAN, 1993).

Até o momento, não há uma abordagem experimental padrão para a investigação de biofilmes, observa-se que cada método tem sido complementar ao outro. Além disso, os conhecimentos advindos de vários estudos envolvendo a aplicação de metodologias combinadas proporcionaram diferentes ensaios sobre a natureza dos biofilmes em diversos micro-organismos (BRANDA et al., 2005). Neste sentido, os sistemas de microplacas são comumente utilizados como forma de investigação qualitativa e quantificativa de biofilmes (CHRISTENSEN et al., 1985). Adicionalmente, neste método um grande número de isolados pode ser rapidamente analisado tornando-o um ensaio padrão no estudo de biofilmes (O''TOOLE & KOLTER, 1998). Neste estudo, observou-se que e a interação entre os diferentes métodos de investigação sobre formação de biofilmes, incluindo a cultura e analise microscópica, foi útil na validação dos resultados, permitindo novas adaptações metodológicas para futuros estudos de biofilmes em *R. equi*.

O aumento do número de isolados de *R. equi* produtores de biofilmes pelo método de cultura suplementado com glicose era esperado. Da mesma forma, outros trabalhos utilizando o enriquecimento com glicose obtiveram resultados superiores em relação à formação de biofilmes (RODRIGUES et al. 2010), concordando com os resultados observados neste estudo. Nos testes empregados nesta pesquisa, o tempo de incubação para formação de biofilme foi de 24 horas; no entanto, nos ensaios de formação de biofilmes por *Rhodococcus ruber* foram necessários quatro dias para a sua produção máxima (MOR & SIVAN, 2008). Isto pode ter gerado resultados falso negativos nos ensaios com *R. equi*. Embora testes com *R. ruber* não tenham demonstrado diferenças de temperatura, fontes de nitrogênio e pH entre os padrões de crescimento séssil e em agregados celulares, outros estudos são necessários para determinar em que superfícies e sob quais condições ambientais *R. equi* produzem biofilmes.

A resistência antimicrobiana mediada por efluxo foi descrita há muito anos, com estudos de resistência à tetraciclina em *Escherichia coli* (MCMURRAY et al., 1980). O aumento das doses de antimicrobianos e/ou desinfetantes no combate às bactérias patogênicas e o uso de metais pesados nas indústrias implicam no desenvolvimento de uma pressão seletiva na

sobrevivência desses agentes. Portanto, em um ambiente diversificado, as células bacterianas podem adquirir resistência e/ou tolerância aos diferentes compostos tóxicos existentes no meio ambiente (WREN, 2000). O efluxo ativo de macrolídeos é comumente mediado pela família MFS (BUTAYE et al., 2003). Este mecanismo favorece as células bacterianas, pois garante que um sistema de efluxo específico seja ativado em presença de uma determinada quantidade de antimicrobiano. Desta forma, o sistema de efluxo pode mediar altos níveis de resistência específicos para diferentes compostos (BUTAYE et al., 2003). Neste trabalho, foi possível observar a ocorrência de sistemas de efluxo em isolados de *R. equi* de diversas origens, incluindo isolados clínicos, de fezes e de solo. Contudo, futuros estudos devem ser conduzidos buscando–se avaliar a presença de bombas de efluxo em isolados de *R. equi* multirresistentes e suas possíveis correlações.

Bactérias em biofilmes podem, simultaneamente, produzir enzimas que degradam antimicrobianos e alvos de baixa afinidade a estes compostos. Ainda, podem super-expressar genes de bombas de efluxo, codificando proteínas de extrusão, as quais aumentam significativamente o espectro de resistência a inúmeros substratos (HØIBY et al., 2010). Por fim, salientamos a possibilidade de ambos os mecanismos abordados neste estudo atuarem conjuntamente em isolados de *R. equi*, tornando-os extremamente resistentes, tanto em sua vida saprofítica quanto patogênica.

5. CONCLUSÕES

Com base nos resultados obtidos neste estudo é possível concluir que:

Os isolados de *R. equi* analisados, especialmente os isolados de fezes, foram capazes de formar biofilme nas condições testadas.

O padrão de desenvolvimento na forma de biofilme proporcionou aos isolados maior resistência aos antimicrobianos testados, o que pode estar contribuindo para o fenômeno de resistência antimicrobiana observado em isolados de *R. equi* nos últimos anos.

O gene *req_39680* está presente nos isolados de *R. equi* de forma uniforme em relação à origem das cepas.

A expressão fenotípica de mecanismo de efluxo está intimamente correlacionada à presença do gene *req_39680*, especialmente nos isolados de origem clínica.

Os dois mecanismos de resistência estudados nesta pesquisa apresentam potencial para favorecer a sobrevivência de isolados de *R. equi*, tanto no ambiente quanto no hospedeiro.

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