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Letícia Trevisan Gressler

Rhodococcus equi E METABOLISMO DO FERRO: ASSOCIAÇÃO COM SUSCEPTIBILIDADE GENÉTICA E SOBREVIVÊNCIA EM MACRÓFAGOS

Santa Maria, RS, Brasil
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Tese apresentada ao Curso de Doutorado 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 título em **Doutor em Medicina Veterinária**

Orientador: Prof[a]. Dr[a] Agueda Castagna de Vargas

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RESUMO

Rhodococcus equi E METABOLISMO DO FERRO: ASSOCIAÇÃO COM SUSCEPTIBILIDADE GENÉTICA E SOBREVIVÊNCIA EM MACRÓFAGOS

AUTOR: Letícia Trevisan Gressler
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A equideocultura é uma atividade em ascensão mundial, responsável pela geração de empregos e renda. No Brasil, em especial no Rio Grande do Sul, encontram-se diversos locais de criação de equinos de alto padrão zootécnico. Embora estes rebanhos estejam sob rigoroso controle sanitário, a ocorrência de doenças respiratórias é causa importante de mortalidade em potros e redução de seu desempenho atlético. Dentre as doenças respiratórias, a rodococose equina, causada pela bactéria *Rhodococcus equi*, é a principal causa de pneumonia nesta categoria animal. *R. equi* está distribuído mundialmente, e cresce como causa de perdas econômicas devido à pneumonia observada em animais jovens. No entanto, medidas preventivas e efetivo controle da enfermidade são ainda desafios a serem alcançados. Na infecção por *R. equi*, o ferro (Fe) apresenta-se como um elemento fundamental não somente para multiplicação da bactéria, mas também, como um determinante para a expressão de fatores de virulência. Estudos têm demonstrado a presença de mecanismos específicos de captação de Fe em *R. equi*, os quais determinam sua sobrevivência tanto durante seu estilo de vida saprófito quanto patogênico. Em contrapartida, como uma forma de imunidade nutricional, mamíferos, entre eles os equinos, diminuem a concentração plasmática de Fe através de sua ligação em proteínas, entre elas, a transferrina (Tf). Neste contexto, esta tese foi elaborada visando contemplar aspectos relacionados ao metabolismo e aquisição de Fe por *R. equi* e sua importância para patogenia da rodococose equina (manuscrito 1), controle e tratamento de infecções por *R. equi* através de drogas com capacidade de modular a disponibilidade de Fe intracelular (manuscrito 2), e susceptibilidade genética à pneumonia por *R. equi* (manuscrito 3), incluindo a avaliação de polimorfismos no gene da Tf equina como fatores de risco relacionados à susceptibilidade e/ou resistência genética à rodococose equina (manuscrito 4). Concluímos que *R. equi* está evoluindo de forma a especializar-se na aquisição e utilização de Fe a partir do hospedeiro, habilidades que devem ser consideradas como pontos chave no desenvolvimento de agentes quimioterápicos. Uma vez que *R. equi* codifica redundantes mecanismos de aquisição e utilização de Fe, é provável que agentes quimioterápicos deverão inibir múltiplos mecanismos ou ser utilizados em combinação. Além disso, o conceito de "imunidade nutricional" pode considerado uma importante estratégia para minimizar a resistência antimicrobiana observada em *R. equi.* Como um exemplo de quimioterápicos associados ao metabolismo de Fe, observados que chloroquine inibir a multiplicação intracelular de *R. equi*, muito provavelmente devido à deprivação de Fe intracelular. No entanto, ainda são necessários estudos avaliando o potencial terapêutico de chloroquine como tratamento alternativo de infecções por *R. equi.* Observou-se, também, importantes regiões cromossômicas positivamente associadas à pneumonia por *R. equi*, as quais parecem possuir genes associados à resposta imune contra patógenos intracelulares. Esta observação nos permite classificar a rodococose equina como uma enfermidade de base poligênica, como postulado por estudos anteriores. Por fim, verificamos que polimorfismos no gene da Tf, inclusive polimorfismos ainda não descritos na literatura, ocorrem em equinos das raças Brasileiro de Hipismo e Puro Sangue de Corrida, criados no Brasil. Existe a ocorrência de dois alelos entre as raças estudas, incluindo animais heterozigotos para estes alelos. Acredita-se que exista uma relação entre variantes de Tf equina e susceptibilidade genética à pneumonia por *R. equi* nas raças analisadas. Em suma, demonstrou-se através de diferentes estudos que a modulação da disponibilidade de Fe pode ser uma forma de controle da rodococose equina.

Palavras-chave: Chloroquine. Equino. Ferro. *Rhodococcus equi*. Susceptibilidade Genética. Transferrina.

ABSTRACT

Rhodococcus equi AND IRON METABOLISM: ASSOCIATION WITH GENETIC SUSCEPTIBILITY AND SURVIVAL WITHIN MACROPHAGES

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Horse breeding industry is an activity in ascension worldwide, and is responsible for generating jobs and income. In Brazil, especially in Rio Grande do Sul state, there are several horse breeding farms with high-standard equines. Although these herds are under strict sanitary control, the occurrence of respiratory diseases is an important cause of mortality in foals and reduced athletic performance. Among the respiratory diseases, equine rhodococcosis, caused by the bacterium *Rhodococcus equi*, is the major cause of pneumonia in foals. *Rhodococcus equi* is worldwide distributed, and have emerged as an important cause of economic losses due to pneumonia in young animals. However, preventive measures and effective control of the disease are still challenges to be reached. In *R. equi* infection, iron (Fe) is classified as an essential element not only for the bacterium multiplication, but also as a key for the expression of virulence factors. Studies have shown the presence of specific Fe uptake mechanisms in *R. equi*, which have been determining its survival in both saprophytic and pathogenic life styles. However, as a type of nutritional immunity, mammals, including horses, reduce the plasmatic concentration of Fe through its binding in proteins, including the transferrin (Tf). In this context, the present thesis was developed to study aspects related to metabolism and acquisition of Fe by *R. equi* and Fe importance in the pathogenesis of equine rhodococcosis (manuscript 1), control and treatment of infections caused by *R. equi* through drugs with capability to reduce the availability of intracellular Fe (manuscript 2), and genetic susceptibility to *R. equi* pneumonia (manuscript 3), including the assessment of polymorphisms in the equine Tf gene as risk factors related to susceptibility and/or resistance to equine rhodococcosis (manuscript 4). We conclude that *R. equi* is evolving to specialize it in the acquisition and utilization of Fe from the host, skills that should be considered as key points for the development of chemotherapeutic agents. Once *R. equi* encodes redundant mechanisms of acquisition and utilization of Fe, it is likely that chemotherapeutic agents will need act on multiple cellular mechanisms or be used in combination. Furthermore, the term "nutritional immunity" may be considered an important strategy to minimize antimicrobial resistance observed in *R. equi*. As an example of chemotherapy associated with iron metabolism, we observed that chloroquine inhibits the intracellular multiplication of *R. equi*, most likely due to intracellular iron deprivation. However, further studies are necessary to evaluate the chloroquine therapeutic potential against *R. equi* infections. We also observed important chromosomal regions positively associated with *R. equi* pneumonia, which seem to possess genes associated with immune response against intracellular pathogens. This observation allows us to classify the equine rhodococcosis as a disease of polygenic basis, as postulated by previous studies. Finally, we found that polymorphisms in the Tf gene, including some not described yet in the literature, occur in Brazilian Sport Horses and Brazilian Thoroughbred Horses. There is the occurrence of two alleles between the breeds studied, including heterozygosis for these alleles. We believe that there is a relationship between equine Tf variants, and genetic susceptibility to *R. equi* pneumonia in the breeds evaluated. Summarizing, we have demonstrated that the modulation of Fe availability may be a useful approach to control the disease.

Keywords: Chloroquine. Equine. Iron. *Rhodococcus equi*. Genetic susceptibility. Transferrin.

SUMÁRIO

1. INTRODUÇÃO

1.1. PNEUMONIA EM POTROS CAUSADA POR *Rhodococcus equi*: DESAFIO PARA EQUIDEOCULTURA MUNDIAL

Em diversas regiões do mundo o cavalo é um importante animal de companhia, esporte, lazer, trabalho e transporte. Deste modo, sua saúde e bem-estar são extremamente valorizados na sociedade atual. Segundo a Organização das Nações Unidas para Agricultura e a Alimentação (FAO), o planeta possui mais de 50 milhões equinos (espécie *Equus caballus*). A liderança na produção pertence aos Estados Unidos da América (EUA), com cerca de 10 milhões de animais, seguido da China, com aproximadamente 6,4 milhões, do México, com 6,3 milhões, e do Brasil, com 5,4 milhões (FAO, 2014). No Brasil, de acordo com o Centro de Estudos Econômicos da Universidade de São Paulo (Cepea), este segmento movimenta cerca de R$ 8,5 bilhões anuais, proporcionando mais de três milhões de empregos diretos e indiretos (CEPEA, 2006). Em resposta à intensificação na produção de equinos observa-se a ocorrência de doenças infectocontagiosas, como a rodococose equina, causada pela bactéria *Rhodococcus equi* (PRESCOTT, 1991).

Mundialmente, inclusive no Brasil, a pneumonia por *R. equi* é a principal causa de morte em potros (GIGUÈRE et al., 2011). No Brasil encontram-se diversos haras destinados à criação de equinos com alto padrão zootécnico, incluindo animais das raças Puro Sangue Inglês (PSI), Brasileiro de Hipismo (BH), Quarto de Milha, Crioula, entre outras. Embora as necessidades de manejo divirjam significantemente entre as raças, cuidados com a sanidade são fundamentais para o sucesso deste tipo de empreendimento. Neste sentido, há o desafio de controlar e prevenir a ocorrência de enfermidades infecciosas, especialmente as do trato respiratório, as quais têm refletido em significativos prejuízos econômicos. Em geral, as perdas econômicas estão associadas aos custos com atendimento médico veterinário, medicamentos (longo período de tratamento), mortalidade (VENNER et al., 2012) e complicações secundárias que podem encerrar prematuramente a vida produtiva do animal (AINSWORTH, 1997). A infecção por *R. equi* representa, ainda, um desafio para saúde pública, uma vez que este patógeno pode causar lesões semelhantes à tuberculose em humanos, especialmente em indivíduos imunocomprometidos, idosos e crianças (PRESCOTT, 1991). Desta forma, ressalta-se o potencial zoonótico de *R. equi*.

1.2. *Rhodococcus equi*: UM PATÓGENO VERSÁTIL E DE DIFÍCIL CONTROLE

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 enterocolite ulcerativa, linfadenite mesentérica, osteomielite, artrite purulenta e linfangite ulcerativa (PRESCOTT, 1991). De forma menos acentuada, *R. equi* pode causar manifestações clínicas como enterite, linfadenite, abortamento, mastite, dermatite e piometra em outras espécies animais, como bovinos, caprinos, bubalinos, cães e gatos (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).

A patogenicidade de *R. equi* está intimamente relacionada à presença de genes que codificam proteínas associadas à virulência (*virulence associated protein,* Vap*)*, os quais encontram-se localizados em um plasmídeo de virulência. O principal antígeno de virulência relacionado à infecção em equinos caracterizado até o momento é a proteína de superfície VapA, codificada pelo gene *vapA* (BYRNE et al., 2001). Observou-se que isolados *vapA*-negativos não possuem capacidade de sobreviver e replicar no interior de macrófagos murinos (TAKAI et al., 1991). Posteriormente, observou-se que cepas de *R. equi vapA*-negativas (sem plasmídeo de virulência) apresentaram reduzida patogenicidade para camundongos e equinos (TAKAI et al., 1995; WADA et al., 1997). A partir do conhecimento destes genes, isolados 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), isolados de virulência intermediária, carreadores da VapB, proteína similar à VapA, porém observada em isolados de *R. equi* provenientes de humanos e de suínos, e avirulentos, que não apresentam plasmídeo relacionado à virulência (RIBEIRO et al., 2005). Devidos à elevada patogenicidade, a

infecção por *R. equi* tornou-se um desafio, especialmente para potros, cujo sistema imune parece não ser capaz de conter a multiplicação de isolados virulentos.

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 com o declínio das taxas de anticorpos adquiridos pela imunidade passiva e com a imaturidade do sistema imunológico (GYLES et al., 2010). Além disso, outros fatores podem estar associados à ocorrência da doença, como alta densidade de animais, sistema de criação intensivo, manejo que predisponha o contato com animais de outras fazendas, e 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 podem contribuir 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 de forma esporádica e em outras é endêmico, resultando em surtos da doença (TAKAI, 1997).

Uma vez que exista suspeita de infecção por *R. equi*, o diagnóstico definitivo pode ser realizado através de isolamento e identificação do agente a partir de amostras de aspirado traqueobronquial, e confirmação por reação em cadeia da polimerase (PCR), identificando-se inclusive a presença do gene *vapA* (GIGUÈRE, 2001). 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 de diagnóstico precoce.

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, azitromicina, e claritromicina, associados a rifampicina (GIGUÈRE, 2000). Esta combinação promove uma ação sinérgica entre os antimicrobianos, resultando em adequada penetração tecidual. Por outro lado, 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 cepas resistentes têm sido relatado (TAKAI et al., 1997; ASOH et al., 2003; NIWA et al., 2006).

O controle e a prevenção de infecções por *R. equi* pode tornar-se difícil devido sobretudo à necessidade de diagnóstico precoce e isolamento de animais infectados. É importante salientar que o diagnóstico precoce auxilia na diminuição da ocorrência da doença clínica, mortalidade e custos com o tratamento (MUSCATELLO, 2012). O emprego da vacinação ainda não é uma opção viável, uma vez que não existe vacina disponível comercialmente.

1.3. BASE GENÉTICA ASSOCIADA À INFECÇÃO POR *Rhodococcus equi*

Embora exista um avanço considerável no entendimento da pneumonia por *R. equi* associada a fatores de risco relacionados ao ambiente e manejo dos animais, pouco se sabe sobre a influência de características do hospedeiro. Acredita-se que estudos direcionados à função imune de animais afetados e não afetados possa ser um ponto importante para explicar o porquê alguns animais desenvolvem a doença, enquanto outros não, mesmo quando submetidos às mesmas condições de criação (CHAFFIN et al., 2003; HALBERT et al., 2006). Neste sentido, alguns autores têm sugerido e demonstrado fortes evidências de uma base genética para susceptibilidade de potros à infecção por *R. equi* (BRANDON et al., 1999; MOUSEL et al., 2003; HALBERT et al., 2006; HORIN et al., 2009; McQUEEN et al., 2014).

Tanto a imunidade inata, através de células de resposta primária, como macrófagos e neutrófilos, como a imunidade adquirida, através de linfócitos e anticorpos secundários, são cruciais contra a infecção por *R. equi*. Mutações nos genes *Casp1* e *IL7R*, relacionados à IL-1 e IL-7, respectivamente, foram associadas à modulação da infecção por *R. equi* em potros (HORIN et al., 2008; HORIN et al., 2009). Recentemente, um importante estudo, realizado com cavalos da raça Quarto de Milha, revelou uma forte associação entre mutações no gene *TRMP2* (envolvido na função de neutrófilos) e a ocorrência de pneumonia por *R. equi* (McQUEEN et al., 2014). Genes envolvidos no transporte de Fe, como a transferrina (Tf) (MOUSEL et al, 2003) e SLC11A1 (HALBERT et al., 2006), também têm sido associados à etiologia genética da infecção por *R. equi* em potros.

Os polimorfismos observados em genes relacionados à resposta imune possuem importante papel biológico, especialmente se houver um sinergismo entre estes genótipos (HORIN et al., 2009). Por ser uma doença complexa, a base genética parece desempenhar um

papel importante na ocorrência da rodococose em equinos (McQUEEN et al., 2014). Em meio aos inúmeros fatores potencialmente associados à ocorrência de pneumonia por *R. equi*, ressaltamos a importância da captação do ferro como fator crucial para o sucesso da sobrevivência de *R. equi* no interior de macrófagos.

1.4. RELAÇÃO ENTRE TRANSFERRINA (Tf) E FERRO (Fe)

A Tf é uma glicoproteína de 80 kDa, sintetizada principalmente no fígado, cuja função é transportar o Fe do plasma para os tecidos, promovendo o crescimento e a diferenciação celular, além da bacteriostase (de JONG et al., 1990). Desta forma, a Tf atua fazendo com que o sangue se torne um meio de cultura inadequado para crescimento bacteriano (AISEN & LISTOWSKY, 1980). Variações genéticas em Tf da espécie humana foram descritas há muitos anos, desde estudos incipientes na década de 50 (SMITHIES, 1957), até estudos sobre polimorfismos e sua influência no metabolismo do Fe (GOTTSCHALK et al., 2000; KASVOSVE et al., 2000). Em animais, a investigação de polimorfismos (bioquímicos) da Tf iniciou na década de 60, incluindo várias espécies, como bovinos, ovinos e equinos (BRAEND & STORMONT, 1964; PUTNAM & ROOP, 1965; SPOONER et al., 1975). Em equinos, foram descritas mais de 15 variantes (alelos) de Tf (F_1, F_2, F_3, F^*, D_1, D_2, G, H_1, H_2, M, O, O^*, R, R^* e E) por meio de estudos bioquímicos e moleculares (BELL et al., 1988; BRAEND & STORMONT, 1964; BRANDON et al., 1999; CHUNG & McKENZIE, 1985; KAMINSKI et al., 1981).

A existência de um grande número de variantes para Tf equina sugere a ocorrência de uma pressão de seleção nesta espécie, que pode estar relacionada à infecção por bactérias detentoras de sofisticados sistemas de captação de Fe a partir da Tf (MOUSEL et al., 2003). Ainda, segundo Mousel et al. (2003), é possível estabelecer se um animal é mais susceptível ou resistente à infecção por *R. equi* baseando-se no tipo de Tf. Estudos preliminares apontam que a presença do alelo F pode resultar em suscetibilidade e a presença do alelo D, em resistência à infecção por *R. equi* (MOUSEL et al., 2003; NEWTON et al., 2007).

O Fe desempenha um papel crítico como transportador de elétrons e como biocatalisador de reações, sendo assim, essencial para a maioria das bactérias (ANDREWS et al., 2003). Na infecção por *R. equi*, o Fe apresenta-se como um elemento fundamental não somente para manutenção da célula, mas também como um limitante para a expressão de fatores de virulência (JORDAN et al., 2003). Embora abundante na natureza, o Fe permanece insolúvel em pH neutro e em ambiente aeróbio. Em mamíferos, o Fe livre é ainda sequestrado

por proteínas, como as lactoferrinas e Tf ou está ligado ao grupo heme. Nestas condições, a quantidade de Fe chega a 10^{-24} M, muito abaixo do necessário para promover o crescimento bacteriano, o qual necessita em torno de 10^{-7} M (BRAUM, 2001). Desta forma, a fim de compensar as baixas concentrações de Fe, algumas bactérias desenvolveram sofisticados meios de capturá-lo (BYRD & HORWITZ, 2000).

1.5. SISTEMAS DE CAPTAÇÃO DE FERRO POR *Rhodococcus equi*

Como parte da resposta inata de fase aguda, os mamíferos respondem às infecções diminuindo ainda mais a concentração plasmática de Fe (RATLEDGE, 2007). Em contrapartida, alguns micro-organismos possuem sistemas que agem como quelantes. Estes sistemas são denominados sideróforos, uma vez que possuem alta especificidade e afinidade por Fe^{3+} (BRAUM, 2001). Existe cinco principais grupos de sideróforos classificados de acordo com suas estruturas bioquímicas: i. hidroxamatos, ii. catecolatos, iii. carboxilatos, iv. compostos heterocíclicos e v. mistos (DRECHSEL & WINKELMANN, 1997). *R. equi* possui *rhequibactin*, um catecolato, e *rhequichelin*, um hidroxamato (MIRANDA-CASOLUENGO et al., 2008; 2012). *Rhequibactin* está relacionado ao estilo de vida saprofítico de *R. equi*, enquanto que *rhequichelin* atua como parte de um processo de regulação de fatores associados à virulência, onde a captação de Fe é um determinante para patogenicidade (MIRANDA-CASOLUENGO et al., 2008; 2012).

A síntese de *rhequichelin* é fortemente aumentada durante as 10 primeiras horas após a infecção de macrófagos por *R. equi*, muito provavelmente devido ao ambiente de restrição de Fe no interior da célula (MIRANDA-CASOLUENGO et al., 2012). Esta observação vem ao encontro de outros estudos que também demonstraram a influência da restrição de Fe na expressão de fatores de virulência em *R. equi* (BENOIT et al., 2002; RUSSEL et al., 2004). De forma similar à ação do sideróforo *mycobactin*, presente em *Mycobacterium tuberculosis* (DE VOSS, 2000), *rhequichelin* é essencial para o crescimento bacteriano no interior de macrófagos, muito provavelmente devido a sua atuação na aquisição de Fe a partir da Tf (MIRANDA-CASOLUENGO et al., 2012).

Além dos sistemas de captação de Fe por meio de sideróforos, algumas bactérias patogênicas possuem receptores de superfície, os quais capturam este micronutriente diretamente da Tf e transportam-no através da membrana externa (GRAY-OWEN & SCHRYVERS, 1995). Estas bactérias, capazes de utilizar a Tf como fonte direta de Fe, possuem uma grande vantagem adaptativa (BRAUM, 2001). Para exemplificar, a bactéria

Actinobacillus pleuropneumoniae expressa receptores de superfície responsáveis pela utilização da Tf como fonte de Fe, sendo este mecanismo essencial para sua sobrevivência no pulmão de suínos (BALTES et al., 2002). Embora ainda sem comprovação científica, as observações verificadas em *A. pleuropneumoniae* podem implicar em significativa vantagem também para *R. equi*, tendo em vista que ambos atuam como patógenos do trato respiratório, causando severo comprometimento pulmonar.

A análise genômica de *R. equi* permitiu a descoberta de inúmeros genes associados à aquisição/regulação de Fe. *R. equi* codifica duas ferritinas bacterianas, importantes no armazenamento de Fe (REQ01640-50), proteínas regulatórias (IdeR e Fur), além de genes codificadores de sideróforos (LETEK et al., 2010). A redundância de sistemas envolvidos na aquisição de Fe em *R. equi* demonstra a importância da disponibilidade de Fe para sobrevivência e patogenicidade deste micro-organismo.

Diante do exposto acima, a seguir serão apresentados quatro manuscritos, os quais abordam metabolismo e aquisição de Fe por *R. equi*, o potencial de um medicamento, cuja ação está relacionada a restrição de Fe intracelular, no tratamento e prevenção de *R. equi*; bem como resultados de dois estudos de susceptibilidade genética, incluindo a análise de polimorfismos presentes no gene da Tf equina. Com exceção do MANUSCRITO 4 que está em fase de preparação, os demais encontram-se submetidos ou em fase de submissão para publicação.

2. MANUSCRITO 1

Review paper

Rhodococcus equi and Iron: Metabolism, Impact on Virulence, and New Therapeutic Approaches

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(Artigo a ser submetido para publicação – Trends in Microbiology)

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Rhodococcus equi and Iron: Metabolism, Impact on Virulence, and New Therapeutic Approaches

Abstract

Rhodococcus equi replicates within macrophages causing a highly prevalent and severe pneumonia in young foals and tuberculosis-like lesions in humans. Iron (Fe) is an essential micronutrient for almost all microorganisms, including *R. equi*. The purpose of this review is to describe how *R. equi* acquires and metabolizes Fe, how Fe is acquired and metabolized by host macrophages, and how this competition determines the fate of *R. equi* within macrophages. We further describe associations between Fe-regulatory genes and susceptibility of foals to *R. equi* pneumonia, and conclude with chemotherapeutic approaches against *R. equi* based on a principle termed *nutritional immunity*. Finally, it is our belief that this knowledge indicates Fe is a key target for novel chemotherapeutic agents against *R. equi* pneumonia.

Key-words: Iron, *Rhodococcus equi*, siderophore, *vapA*, Gallium, Chloroquine

1. Introduction

Iron (Fe) is an essential element for both pathogens and their hosts. In bacteria, Fe is a coenzyme or enzyme activator of ribonucleotide reductase, a key enzyme for DNA synthesis. Iron also participates in a large number of cellular processes, such as oxygen transport, ATP generation, and cell growth and proliferation (Andrews, 1998). This micronutrient modulates immune effector mechanisms, such as cytokine activities, nitric oxide (NO) formation or immune cell proliferation, and, consequently, participate of host immune surveillance (Weiss, 2005). High concentrations of Fe, however, can be toxic to cells due to generation of oxidative radicals. For this reason, Fe homeostasis is precisely regulated within the cell, resulting in practically no free Fe either extracellularly or intracellularly. Because Fe is essential for survival of microorganisms, its deficiency can kill them by a nutritional-

starvation pathway. Thus, Fe acquisition and utilization is possibly the major determinant of success of a microorganism's ability to maintain itself within a host (Colin & Dover, 2000).

Like other intracellular pathogens such as *Mycobacterium* spp., *Rhodococcus equi* can survive and replicate inside macrophages (Hondalus, 1994), where Fe is not a freely-available nutrient (Wooldridge & Williams, 1993). Over time, both bacteria and host have developed systems to compete with each other for Fe in a coevolution scenario. As a multi-host pathogen,

R. equi has evolved to obtain Fe by multiple methods, according to selective pressures for iron restriction by the host (Ocampo-Sosa et al., 2007). Thus, *R. equi* has redundant mechanisms to access Fe from the host (Letek et al., 2010), such that Fe can be acquired by another mechanism when a given mechanism is blocked. The ability of *R. equi* to acquire Fe from the host can therefore be considered a determining in the organism's ability to evade the immune system and cause disease in the host.

Pneumonia caused by *R. equi* remains highly prevalent in many countries. In humans, *R. equi* cause tuberculosis-like lesions primarily among those who are immune-compromised. Highly effective methods for preventing *R. equi* pneumonia in foals are lacking, and the current standard for treatment (a macrolide in combination with rifampin) is not completely effective and is threatened by the emergence of resistance of *R. equi* to macrolides (Fines et al., 2001; Giguère et al., 2010; Niwa & Lasker, 2010; Venner et al., 2012; Burton et al. 2013; Cisek et al., 2014; Anastasi et al., 2015). The roles of humoral and cellular immunity to combat *R. equi* infection have been well studied (reviewed in Dawson et al., 2010). The concept of *nutritional immunity*, which has received relatively little attention, should be considered as an alternative to prevent and/or combat *R. equi* infection. The administration of excess Fe increases the virulence of numerous pathogens in animal models, highlighting the impact of micronutrients on host susceptibility to infection and disease (Barton et al, 1999;

Crosa et al., 2004). Conversely, Fe deficiency was found protecting young children against severe *Plasmodium falciparum* malaria (Gwamaka et al., 2012). The purpose of this report is to review current knowledge and recent advances in our understanding of Fe metabolism by *R. equi*. We hope to demonstrate that targeting availability and use of Fe by *R. equi* represent an effective approach for the identification and development of novel chemotherapeutic agents effective against *R. equi* pneumonia.

2. Iron: general characteristics, host detoxification mechanisms, and impact on microorganisms growth

Fe is an essential nutrient for all living cells, except perhaps for the lactic acid bacteria where manganese and cobalt are used in place of Fe (Guerinot, 1994; Weinberg, 1997). Under physiological condition, Fe can exist in either the reduced ferrous (Fe^{2+}) form, the principal ionic species, or the oxidized ferric (Fe^{3+}) form. The redox potential of Fe^{2+}/Fe^{3+} ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$) makes it a catalytic center or an electron carrier. As a result, Fe is essential for several biological processes, such as cell respiration, DNA biosynthesis, and regulation of gene expression (Andrews 1998). Despite its vital role, Fe excess beyond that needed for cellular homeostasis can cause damage. Excess Fe results in degradation of lipids, DNA, and other important cellular molecules. Moreover, Fe^{2+} can react with metabolically generated hydrogen peroxide to yield toxic hydroxyl radicals (Ratledge & Dover, 2000).

To counter the toxic potential of iron, cells have evolved several protective mechanisms such as oxidation of excess Fe^{2+} to Fe^{3+} state, binding of Fe by serum proteins, and storage of Fe in ferritin-like proteins. Oxidation of excess Fe^{2+} to Fe^{3+} is crucial to prevent damage to the body. Thus, at aerobic conditions and pH 7, Fe^{2+} is oxidized primarily as Fe^{3+}, which aggregates into ferric hydroxide (aqueous hydroxyl) complexes, with solubility around 10^{-9} M (Ratledge & Dover, 2000). The binding of Fe to serum proteins occurs following intestinal absorption of

dietary Fe. After absorption into circulation, Fe is sequestered into host glycoprotein transferrins (Tfs), such as serotransferin (Tf) and lactotransferrin/lactoferrin (Lt). In humans, once free Fe is in equilibrium with Fe-binding proteins its concentration drops to 10^{-24} M (Thorstensen and Romslo 1990). In addition to the Tfs, Fe can be found in hemoglobin or ferritin. Almost 80% of the Fe in the body is contained in the heme of hemoglobin and myoglobin, and 15% is deposited in ferritin (Braun, 2001). Ferritin is the principal storage depot of Fe in the body. Ferritin has a spherical shape in which Fe is centrally stored; Fe is deposited in ferritin during conditions of iron overload and is released from ferritin during total body Fe deficiency (Andrews, 1998).

These mechanisms for preventing Fe toxicity also function to ensure that bacteria encounter a period of Fe starvation upon entering their hosts. In health, the cellular concentration of free Fe is too low for microorganisms to survive because 10^{-7} M Fe^{3+} is required to support bacterial growth (Braun, 2001). Not surprisingly, bacteria have acquired effective mechanisms for addressing Fe restriction by the host: Fe acquisition by cognate receptors using low molecular weight Fe chelators, termed siderophores; and, receptor-mediated Fe acquisition from host proteins. Additionally, some bacteria possess ferritin-like proteins for Fe storage. There are at least 3 types of ferritin-like proteins found in bacteria: bacterial ferritin, bacterioferritin, and dodecameric ferritin, which are related to the ferritins found in eukaryotes (Smith, 2004). Below we will briefly present the general mechanisms by which bacteria acquire Fe, and summarize current knowledge of these mechanisms in *R. equi*.

3. Potential systems of iron acquisition by *R. equi*

Bacterial Fe acquisition/uptake systems can be divided into 3 types: heme acquisition systems; siderophore-based systems; and, Tf/Lf receptors. There is a more detailed description available for the components of these systems in Gram-negative than in Gram-positive bacteria. In Gram-negative bacteria, all Fe acquisition pathways require an outer

membrane receptor, a periplasmic binding protein, and an inner-membrane ABC transporter (Guerinot, 1994; Krewulak & Vogel, 2008). In Gram-positive bacteria, the Fe uptake systems are less complex because these bacteria have no outer membrane such that the separation between bacterial cytoplasm from its environment is due to only the cell wall. Therefore, in Gram-positive bacteria, Fe uptake involves basically an inner membrane-anchored binding protein (Guerinot, 1994; Krewulak & Vogel, 2008). Although it is well established that *R. equi* scavenge Fe from its host for survival, the pathways behind this topic are still poorly elucidated.

Heme acquisition systems typically involve specific receptors on the bacterial surface that recognize either free heme or heme bound to hemoproteins such as hemoglobin (Hb) or hemopexin. Heme is removed from hemoproteins by surface proteins and transported through the cell wall of the bacteria into the cytoplasm. Bacterial pathogens can also elaborate secreted heme-scavenging molecules that remove heme from host hemoproteins; these molecules, known as hemophores, are functionally analogous to siderophores but target heme instead of Tfs (Wandersman & Delepelaire, 2004). Once inside the cell, Fe is released from heme by the action of heme oxygenases or reverse ferrochelatase activity (Wilks, 2002).

Rhodococcus equi may encounter heme or Hb at both intracellular and extracellular sites of infection. Once macrophages degrade a significant amount of senescent red blood cells (Soe-Lin et al., 2009), *R. equi* within macrophages may interact with heme by its diffusion into the phagosome, or by a direct contact with heme or Hb released from erythrocytes, similar to what occurs in mycobacterial heme acquisition systems (Tullius et al., 2011). Although in a less pronounced manner than mycobacterial species, *R. equi* (both virulent and avirulent strains) are able to use both heme and Hb as source of Fe (Miranda-CasoLuengo et al., 2005). To date, there is no specific heme surface-receptor described for *R. equi*, however, an Fe-dependent transport system has been reported as being necessary for Fe

acquisition from heme and Hb. The Fe-regulated *iupABC* operon was the first transport system associated with Fe acquisition in *R. equi* (Miranda-CasoLuengo et al., 2005). Whether this operon contributes to transport of heme and Hb into the cytoplasm remains to be determined; however, the disruption of *iupA* resulted in failure of *R. equi* to utilize heme and Hb as source of Fe. In these settings, *R. equi* encodes a putative heme oxygenase protein (REQ_22910) (Letek et al., 2010), used to release Fe from heme after it is shuttled into the cytoplasm. Further studies are needed to confirm that *R. equi* is able to acquire Fe from heme and Hb, and the impact of this source of Fe for *R. equi*.

Another important mechanism by which bacteria acquire Fe is use of siderophore-based systems. Siderophores (from the Greek: "iron carriers") are defined as relatively low molecular weight, ferric ion-specific chelating agents elaborated by microorganisms growing under Fe stress (Guerinot, 1994). These compounds scavenge Fe from the environment and make it available to microbial cell. Because of their high affinity for Fe, with an association constant > 10^{50}, siderophores enable bacteria to compete successfully with Tfs for Fe-bound (Bullen & Griffiths, 1999). While the first mycobacterial siderophore (mycobactin) was isolated and purified in the mid 1950's (Francis et al., 1949; 1953), the first siderophore (rhequibactin) in *R. equi* was identified only by 2008 (Miranda-CasoLuengo et al., 2008).

Rhequibactin is a catecholate siderophore which is likely transported by the IupABC transporter system (Miranda-CasoLuengo et al., 2005). Unlike Fe acquisition from heme or Tfs-loaded proteins, which do not reach the cytoplasm, siderophore-Fe^{3+} complexes are taken up through the outer membrane and into the cell via highly specific siderophore-mediated transport systems (Ferguson et al., 1998; Stintzi et al., 2000). The *iupABC* operon described for *R. equi* is highly similar to other siderophore uptake systems, and its expression, particularly *iupA* transcripts, is significantly up-regulated in *R. equi* grown under Fe-depleted

conditions (REF). Whether the IupABC transporter system translocates rhequibactin-Fe complexes into the *R. equi* cytoplasm has not yet been definitively determined.

Interestingly, disruption of either the *iupABC* operon or rhequibactin failed to inhibit *R. equi* multiplication within macrophages both *in vitro* and *in vivo* (Miranda-CasoLuengo et al., 2005; 2008). Based on these findings, and through bioinformatics investigation of the *R. equi* genome, the laboratory of one of the authors (Miranda-CasoLuengo) concluded that a second siderophore contributed to the intracellular growth of *R. equi* (Miranda-CasoLuengo et al., 2008). In 2012, their laboratory described an hidroxamate siderophore (rhequichelin) required for *R. equi* growth within macrophages (Miranda-CasoLuengo et al., 2012). Additionally, disrupting biosynthesis of rhequichelin attenuated *R. equi* growth in mice ((Miranda-CasoLuengo et al., 2012).

Although *R. equi* is capable of acquiring Fe from bovine holotransferrin (bHTf; 99% Fe-saturated), bovine lactoferrin (bLf; 17% Fe-saturated), and bovine apotransferrin (bATf; 0% Fe-saturated) after its Fe-binding in media containing the 2,2'-dipyridyl (DIP) chelator (Jordan et al., 2003), no specific Tfs receptors have been reported for *R. equi*. Several *vap* genes, among them, *vapA*, *vapC*, *vapD*, *vapE*, *vapF*, *vapG*, and *vapH*, are up-regulated in an Fe-deprived environment (Benoit et al., 2002; Jordan et al., 2003; Ren & Prescott, 2003). To date, the functions of the proteins encoded by these *vap* genes remain undefined. The identification and characterization of specific Tf/Lf receptors in *R. equi* could significantly enhance our understanding of the mechanisms by which this pathogen establishes itself in the host.

4. Iron homeostasis in *R. equi*

The regulation of Fe concentration is essential to survival of both bacterial and host cells (see host detoxification mechanisms previously described in section 2). In bacteria, the

regulation of Fe includes sensing its cellular concentration and responding by modifying its uptake, storing excess Fe for times of starvation, and possibly regulating Fe efflux (Rodriguez, 2006). Because of the extremely tight regulation of Fe homeostasis, there is virtually no free Fe in a living bacterial cell (Wandersman & Delepelaire, 2004). In order for pathogens to implement their intricate systems of Fe acquisition and utilization, they first have evolved to sense Fe depletion as a marker of vertebrate tissue. After bacterial entrance into a vetebrate cell, Fe solubilization and metabolism are regulated by specific membrane-bound receptors and the bacterial ferritins and bacterioferritins (Cabiscol et al., 2000). This Fe sensing typically involves 2 levels of Fe regulation present in *R. equi* and discussed below.

The first level of Fe sensing uses an Fe-dependent transcriptional repressor known as Fur (ferric uptake regulator). Fur binds to target sequences in the promoter of Fe-regulated genes and represses their expression in the presence of Fe; in the absence of Fe, Fur-mediated repression is removed and the genes are transcribed (Ratledge & Dover, 2000). The genomic sequencing of *R. equi* 103S enabled the identification of 2 Fur-like regulators (REQ_04740-*furA* and REQ_29130-*furB*) that might contribute to Fe/metal homeostasis (Letek et al., 2010).

In addition to Fur, many Gram-positive bacteria express an extra Fe-dependent repressor belonging to the DtxR family. The first DtxR family-member identified was the diphtheria toxin produced by *Corynebacterium diphtheria* (Bullen & Griffiths, 1999). Following binding of Fe^{3+}, these proteins bind to their target DNA sequences as a homodimer, resulting in repression of genes involved in processes ranging from Fe acquisition to expression of virulence factors (Tao et al., 1994). A homologue gene, *ideR*, also controls expression of virulence factors in *Corynebacterium diphtheria* and *M. tuberculosis* (Tao et al., 1994; Dussurget et al., 1996).

R. equi also express the *ideR* gene, which encodes an Fe-dependent regulatory protein (IdeR – 29 kDa) (Boland & Meijer, 2000). When Fe is present in the environment it binds to the regulatory protein IdeR. This complex then binds to the promoter region of the genes for the siderophores and acts as a repressor, stopping the transcription of siderophore genes. Under Fe limited conditions, however, Fe does not bind to IdeR such that IdeR does not repress the genes responsible for synthesis of siderophores (Dussurget et al., 1996). In *R. equi*, IdeR appears to regulate plasmid-borne genes, including *vapA*. IdeR represses *vapA* expression, a gene which is significantly up-regulated when environmental Fe is reduced (Ren and Prescott 2003). A putative IdeR binding site overlaps the *vapA* promoter, suggesting that *vapA* is a member of IdeR regulon in *R. equi* (Ren and Prescott 2003). The role of VapA in Fe homeostasis systems of *R. equi* remains to be determined, however.

The second level of Fe sensing is related to storage by the cell, which prevents Fe use by non-essential enzymes during periods of starvation (Masse et al., 2007). The Fur-Fe^{2+} complex activates genes for removal of excess and potentially harmful Fe^{2+}ions from the bacterial cytosol (Ratledge & Dover, 2000). The Fe^{2+} is oxidized to Fe^{3+} and is stored in the bacteria as ferritin or bacterioferritin (Smith, 2004). Advantageously, *R. equi* likely stores intracellular Fe via 2 bacterioferritins (REQ_01640-50) and a Dps/ferritin-like protein (REQ_14900) (Table 1).

Table 1. Ferritin-like compounds in *Rhodococcus equi*

Gene ID	Gene length	Location	Product ID	Product length	Mass of polypeptide
REQ_01640	549 bp	167,219 - 167,767	Bacterioferritin	182 aa	20.425 kD
REQ_01650	546 bp	167,926 - 168,471	Bacterioferritin	181 aa	20.145 kD
REQ_14900	540 bp	1,546,827 - 1,547,366	Dps-like bacterioferritin	179 aa	19.402 kD

*Source: NCBI (http://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/).

5. Iron homeostasis in macrophages and its impact on *R. equi* survival

At the same time as *R. equi* codifies important Fe-regulation systems, such as Fur, IdeR, and ferritin-like compounds, *R. equi* needs to replicate within macrophages, which have their own Fe homeostasis systems. Maintaining eukaryotic cellular Fe content requires precise mechanisms for regulating its uptake, storage, and export (Leon-Sicairos et al., 2015). It is thus important to consider the mechanisms coordinating Fe metabolism in macrophages and how these mechanisms impact intracellular survival and replication.

Macrophages have a crucial role in the host's Fe homeostasis by supplying Fe for erythropoiesis. This Fe is derived by recycling from senescent red blood cells that are phagocytosed by macrophages (De Domenico et al., 2008). This recycled Fe is transported from the lumen of the phagosome into the cytosol by the macrophage protein 1 transporter (Nramp1) (Hentze et al., 2010). The Nramp1 protein is an integral membrane protein expressed exclusively in the lysosomal compartment of monocytes and macrophages. After phagocytosis of bacteria, Nramp1 is targeted to the membrane of the microbe-containing phagosome, where it may modify the intraphagosomal milieu to affect microbial replication (Canonne-Hergaux et al., 1999). Consequently, Nramp1 contributes to defense against infection, especially by intracellular pathogens, by extruding Fe from the phagosome (Canonne-Hergaux et al., 1999; Russel, 2001). Interestingly, dramatic down-regulation of *NRAMP1* transcription was observed in equine macrophages following infection with *R. equi* (Watson et al. 2002).

Fe that is neither utilized by the cell nor stored in ferritin is pumped out of the cell by the exporter ferroportin (Cherayil, 2011). In addition to the Fe obtained by recycling of red blood cells, macrophages obtain this micronutrient from Tf via receptor-mediated endocytosis. Currently, 3 receptors for Tf have been identified in macrophages: the high-affinity receptors TfR1 and TfR2 (Fuchs, et al., 1998), and the lower-affinity receptor

GAPDH (Raje et al., 2007). Internalized Fe (such as that bound to Tf) is released in the acidic environment of endolysosomal compartments and is transported into the cytosol via Nramp2 (Gruenheid et al., 1999; Cherayil, 2011).

Remarkably, slight changes in the Fe intracellular homeostasis can alter macrophage differentiation. Fe is a key component of macrophage polarization, a process by which macrophage function is modified in response to environmental signals such as cytokines (Gaetano et al., 2010). Two distinct states of polarized activation for macrophages have been defined and characterized: the classically activated (M1) macrophage phenotype, and the alternatively activated (M2) macrophage phenotype (Gordon and Taylor 2005; Sica & Mantovani et al. 2012). The M1 macrophages are characterized by an *Fe storing profile*, resulting in high levels of pro-inflammatory cytokines, production of NO and oxygen intermediates, and microbicidal/bacteriostatic effects that contribute to resistance to intracellular pathogens. Conversely, the M2 macrophages show an *Fe release profile*, resulting in parasite clearance and immunoregulatory functions, such as immunosuppression and tissue repair (Gordon and Taylor 2005; Mantovani et al. 2002; Corna et al., 2010).

The limited availability of intracellular Fe contributes to the ability of macrophages to control growth of intracellular pathogens, facilitating microbicidal functions. Corna et al. (2010) suggest that this function is primarily associated with M1-polarized macrophages, which have limited ability to recycle Fe and a restricted pool of labile Fe. Consistent with this line of reasoning, von Bargen et al. (2011) propose that macrophage activation and NO synthesis likely regulate Fe in the following ways: (i) generating macrophages more capable of *storing Fe* in a manner that cannot readily be accessed by *R. equi*; (ii) preventing Fe from being scavenged by bacterial siderophores; and, (iii) inactivating a host-driven pathway to directly supply Fe to the intraphagosome space.

6. Association of susceptibility to *R. equi* pneumonia and Fe- acquisition genes

As indicated previously, pathogens and their hosts have long battled for Fe. In addition to the molecular mechanisms that contribute to this competition, recent findings indicate the competition for Fe might be an important co-evolutionary force (Barber & Elde, 2014). The genetic basis for susceptibility/resistance to *R. equi*, recently reviewed by McQueen et al. (2015), has received increased attention in recent years. Here, we summarize associations between susceptibility to *R. equi* pneumonia in foals and polymorphisms in the *Tf* gene and the solute carrier family 11 member 1 (*SLC11A1)* gene (which encodes the Nramp1 protein).

The *Tf* gene is highly polymorphic in horses. Mousel et al. (2003) propose that the SNPs may be the result of evolutionary selection in which, once *R. equi* evolved to compete for Fe, the horse evolved new Tf variants to resist the pathogen. Although the functional significance of the *Tf* polymorphisms is yet unknown, Brandon et al. (1998) suggest that they might lead to functional differences in Fe-binding or receptor-binding ability, providing an evolutionary advantage to individuals with specific Tf variants. In this way, possessing the *Tf* D allele has been associated with greater resistance to *R. equi* pneumonia while the *Tf* F allele has been associated with increased susceptibility (Mousel et al., 2003), and equine respiratory disease in general (Newton et al., 2007).

Polymorphisms in the *SLC11A1* gene also have been associated with altered susceptibility to tuberculosis (Backwell et al., 2003; Moller & Hoal, 2010). The ability of Nramp1 to lower phagosomal Fe concentrations influences the survival and growth of several intracellular pathogens, such as *Myocbacterium bovis* BCG, *Salmonella typhimurium*, and *Leishmania donovani* (Bradley, 1977; Skamene et al., 1982). Although *Nramp1* gene knockout mice were not more susceptible to infection with *R. equi* (Cohen et al., 2004), polymorphisms in the *SLC11A1* gene were associated with susceptibility to *R. equi* pneumonia among Arabian foals (Halbert et al. 2006). Although the reasons for this

discrepancy are unknown, one explanation is that the murine infection model does not recapitulate the disease in foals.

Under natural conditions, the host response to infection in farm animals is multifactorial and involves the complex interaction between the genomes of the host and the pathogen and the environment (Caron et al., 2004). Thus, pneumonia caused by *R. equi* can be considered multifactorial, and it is likely that both *Tf* and *SLC11A1* genes contribute to susceptibility to infection with *R. equi* in a polygenetic manner (McQueen et al., 2015). Additionally, other genes associated with Fe metabolism also might contribute to genetic susceptibility to *R. equi*. For example, the *SLC40A1*gene would be a reasonable candidate because it encodes the Fe transport protein ferroportin 1 that influences susceptibility to tuberculosis (Baker et al., 2012).

7. Strategies for impairing iron metabolism by *R. equi*

There are a large variety of antimicrobial strategies that act by interfering with microbial acquisition and utilization of Fe, such as the "Trojan horse" strategy, in which drugs are transported into the bacteria via Fe-uptake pathways, and the use of chemical compounds that reduce Fe availability in the cell. Both the semi-metal element gallium (Ga) and the chemotherapeutic compound chloroquine (CQ) have been found to have activity against intracellular *R. equi*.

In the context of *R. equi*, Ga is thought to act to disrupt *R. equi* Fe metabolism and exploit the Fe-restricted environment in macrophages using a "Trojan horse" strategy (Figure 1). Because of chemical similarity between Ga^{3+} and Fe^{3+}, trivalent Ga can substitute for trivalent Fe in many biologic systems, resulting in inhibition of Fe-dependent processes crucial for survival (Hubbard et al., 1986; Chitambar et al., 1988). Like Fe, Ga enters mammalian cells, including macrophages, via both Tf-dependent and Tf-independent uptake

mechanisms (Chitambar & Zivkovic, 1987; Olakanmi et al., 1994). The ability of Ga to inhibit growth of intracellular bacteria by interfering with bacterial Fe metabolism was first established for *Mycobacterium* spp. (Olakanmi et al., 2000). Subsequently, several studies have showed the ability of Ga to inhibit *R. equi* growth either *in vitro* or *in vivo* experiments (Chaffin et al., 2012; Cohen et al., 2015; Coleman et al., 2010; Harington et al., 2006).

Martens et al. (2007a) observed that gallium maltolate (GaM) inhibited growth or killed *R. equi* within infected macrophages. These authors demonstrated that administration of GaM to neonatal foals resulted in Ga serum concentrations (Chaffin et al., 2010) considered sufficient to suppress growth or kill *R. equi* in macrophages based on their previous results (Martens et al., 2007b). Martens and collaborators (2010) also demonstrated that Ga appeared safe when administered orally to foals (Martens et al., 2010). Although oral administration of a methylcellulose formulation of GaM (GaM-MCF), at a dosage of 30 mg/kg once daily for 28 days, failed as a chemoprophylactic against *R. equi* pneumonia in foals (Chaffin et al., 2011), GaM-MCF administered at the same dose was equivalent to macrolides for treating subclinical *R. equi* pneumonia (Cohen et al., 2015). Whether GaM-MCF might have similar efficacy to the combination of a macrolide and rifampin for treatment of clinical *R. equi* pneumonia remains to be determined. It has been suggested that use of GaM is an alternative to macrolides and rifampin might reduce the selection pressure associated to macrolide-resistance in *R. equi*, at least for treatment of subclinical disease (Cohen et al. 2015). Of note, GaM is similarly effective in vitro against macrolide- and rifampin-resistant isolates (Coleman et al., 2010).

Chloroquine (CQ) is a weak base that interferes with intracellular Fe metabolism in a variety of cell-types. This compound crosses the cell membranes readily in its unprotonated form and subsequently becomes protonated intracellularly (Krogstad & Schlesinger, 1987). Once in the cell, CQ raises the pH of lysosomal and endocytic vesicles containing Fe-bound

Tf and impairs the acid-pH-dependent dissociation of Fe (Núñez et al., 1990). By this mechanism, CQ suppresses growth of several intracellular microorganisms including *Legionella pneumophila* (Byrd & Horwitz, 1992), *Paracoccidioides brasiliense* (Dias-Melicio et al., 2007), and *Francisella tulerensis* (Fortier et al., 1995). Based on this knowledge, we are currently evaluating the effectiveness of CQ against *R. equi*. We have observed that CQ inhibits *R. equi* survival within murine and foal alveolar macrophages, likely by an Fe-deprivation-dependent mechanism (unpublished data) (Figure 1).

The use of Fe chelators as therapeutic agents against pathogens is also a strategy widely employed. These compounds, e.g. deferoxamine mesylate (DFO), work as Fe-chelators by binding free Fe in a stable complex. Antimalarial activity of DFO has been demonstrated in animal and human infection models (Gordeuk et al., 1992; Hershko et al., 1992). *Pneumocystis carinii*, an important cause of pneumonia in immunocompromised individuals, also has been shown to be susceptible to DFO treatment (Weinberg, 1994). Nevertheless, use of DFO against *R. equi* is not recommend because we have shown that *R. equi* can acquire and use Fe from DFO (Jordan et al., 2003). An alternative approach would be to assess the efficacy and safety of the Fe chelator 2,2-dipyridyl (DIP), because *R. equi* is unable to use DIP as source of Fe (Jordan et al., 2003).

Another potential therapeutic strategy is use of siderocalin-like compounds. Vertebrates produce siderocalin, also referred to as lipocalin-2 or neutrophil gelatinase-associated lipocalin (NGAL) (Goetz et al., 2002). Siderocalin is secreted by neutrophils at sites of infection. This molecule sequesters siderophore-Fe complexes, thereby preventing bacterial uptake of Fe. Siderocalin has been shown to bind to a different family of siderophores, including the carboxymycobactins, produced by *M. tuberculosis* (Holmes et al., 2005). Siderocalin inhibits multiplication of *M. tuberculosis in vitro* and *in vivo* (Saiga et al. 2008). To date, there are no data about effects of siderocalin against *R. equi* siderocalin-

mediated. Interestingly, siderocalin also binds to endogenous mammalian siderophores, changing the intracellular Fe levels rather than directly binding bacterial siderophores (Yang et al., 2002; Richardson, 2005).

8. Conclusions and future concepts

Although much remains to be learned regarding Fe metabolism by *R. equi*, there is clear evidence that Fe availability and acquisition determines the fate of *R. equi* in the host. It is likely that *R. equi* has evolved to better obtain Fe from the host, such that Fe acquisition mechanisms should be considered a key target for the design of novel chemotherapeutic agents. Because *R. equi* encodes redundant mechanisms to provide itself with Fe, such chemotherapeutic agents will likely have to either inhibit multiple systems of Fe acquisition or be used in combination. Finally, in view of the importance of this micronutrient, we suggest that the concept of *nutritional immunity* be considered as a strategy to minimize *R. equi* antimicrobial resistance.

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References

Allen, C. E. and Schmitt, M. P. (2009) HtaA is an iron-regulated hemin binding protein involved in the utilization of heme iron in *Corynebacterium diphtheriae*. J. Bacteriol. 191, 2638-2648.

Anastasi, E., et al. (2015) Novel transferable erm (46) determinant responsible for emerging macrolide resistance in *Rhodococcus equi.* J Antimicrob Chemother. DOI: doi: 10.1093/jac/dkv279

Andrews, S.C. (1998) Iron storage in bacteria. Adv. Microbial Physiol. 40, 281-351.

Barber, M.F. and Elde, N.C. (2014) Escape from bacterial iron piracy through rapid evolution of transferrin. Science 346 (6215), 1362-1366

Barton, C.H., et al. (1999) Nramp 1: a link between intracellular iron transport and innate resistance to intracellular pathogens. Journal of Leukocyte Biology 66 (5), 757–762

Baker, M.A. et al. (2012) Polymorphisms in the gene that encodes the iron transport protein ferroportin 1 influence susceptibility to tuberculosis. J. Infect. Dis. 205(7), 1043-1047

Benoit, S. et al. (2002). H2O2, which causes macrophage-related stress, triggers induction of expression of virulence-associated plasmid determinants in *Rhodococcus equi*. Infect. Immun. 70, 3768–3776

Bradon, R.B. et al. (1999). Single nucleotide polymorphisms in the equine transferrin gene. Anim Genet. 30(6), 439-443

Bradley, D.J. (1977) Regulation of Leishmania populations within the host. II. genetic control of acute susceptibility of mice to *Leishmania donovani* infection. Clin. Exp. Immunol. 30(1), 130-140

Braun, V. (2001) Iron uptake mechanisms and their regulation in pathogenic bacteria. International J. Med. Microbiol. 291, 67-79

Boland, C.A. and Meijer, W.G. (2000) The iron dependent regulatory protein IdeR (DtxR) of *Rhodococcus equi*. FEMS Microbiology Letters, 191, 1-5

Bullen, J.J. and Griffiths, E. (1999) Iron and infection: molecular, physiological and clinical aspects. New York: John Wiley and Sons

Byrd, T.F. and Horwitz, M.A. (1991) Chloroquine Inhibits the Intracellular Multiplication of *Legionella pneumophila* by Limiting the Availability of Iron. J. Clin. Invest. 88, 351-357

Cabiscol, E. et al. (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. 3, 3-8

Colin, R. and Dover, L.G. (2000) Iron Metabolism in Pathogenic Bacteria. Annual Review of Microbiol. 54, 881-941

Caron, J. et al. (2004) Genetic susceptibility of infectious diseases linked to NRAMP1 gene in farm animals. In: Cellier M, Gros P, editors. The Nramp family. Eurekah.com and Kluwer Academic/Plenum Publishers

Carn, J. et al. (2000) Genetic Susceptibility to Infectious Diseases Linked to NRAMP1 Gene in Farm Animals. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-. Available from: http://www.ncbi.nlm.nih.gov/books/NBK6283/

Cisek, A.A. et al. (2014) Antimicrobial resistance in *Rhodococcus equi*. Acta Biochimica Polonica, 61 (4), 633-638

Chaffin, M.K. et al. (2010) Pharmacokinetics of an orally administered methylcellulose formulation of gallium maltolate in neonatal foals. J. Vet. Pharmacol. Ther. 33, 376–382.

Chaffin, M.K. et al. (2011) Evaluation of the efficacy of gallium maltolate for chemoprophylaxis against pneumonia caused by *Rhodococcus equi* infection in foals. AJVR 72 (7), 945-57

Cherayil, B.J. (2011) The role of iron in the immune response to bacterial infection. Immunol Res. 50(1), 1–9

Chitambar, C.R. et al. (1988) Inhibition of leukemic HL60 cell growth by transferrin-gallium: effects on ribonucleotide reductase and demonstration of drug synergy with hydroxyurea. Blood 72(6), 1930-1936

Chitambar, C.R. and Zivkovic, Z. (1987) Uptake of gallium-67 by human leukemic cells: demonstration of transferrin receptor-dependent and transferrin-independent mechanisms. Cancer Res. 47(15), 3929-3934

Coleman, M. et al. (2010). In vitro antimicrobial activity of gallium maltolate against virulent *Rhodococcus equi*. Vet. Microbiol. 146, 175–178

Corna, G. et al. (2010) Polarization dictates iron handling by inflammatory and alternatively activated macrophages. Haematologica 95(11), 1814-1822

Crosa, J.H. et al. (2004) Iron transport in bacteria. Washington (D.C.): ASM Press. 499 p.

Cohen, N.D. et al. (2004) *Nramp1* deletion does not confer susceptibility to *Rhodococcus equi* infection in mice. Immunogenetics 56, 65–67

Cohen, N.D. et al. (2003) Epidemiologic study of results of pulsed-field gel electrophoresis of isolates of *Rhodococcus equi* obtained from horses and horse farms. Am. J. Vet. Res. 64, 153–161

Cohen, N.D. et al. (2015) Gallium Maltolate as an Alternative to Macrolides for Treatment of Presumed *Rhodococcus equi* Pneumonia in Foals. J. Vet. Intern. Med. 29, 932–939

Dawson, T.R. et al. (2010) Current understanding of the equine immune response to *Rhodococcus equi*. An immunological review of *R. equi* pneumonia. Vet. Immunol. Immunopathol. 135(1-2), 1-11

Dias-Melicio, L.A. et al. (2006) Chloroquine Inhibits *Paracoccidioides brasiliensis* Survival within Human Monocytes by Limiting the Availability of Intracellular Iron. Microbiol. Immunol. 50(4), 307–314

Dias-Melicio, L.A. et al. (2007) Chloroquine is therapeutic in murine experimentalmodel of paracoccidioidomycosis. FEMS Immunol. Med. Microbiol. 50, 133–143

De Domenico, I. et al. (2008) Regulation of iron acquisition and storage: consequences for iron-linked disorders. Nat. Rev. Mol. Cell. Biol. 9(1), 72-81

Dussurget, O. et al. (1996) ideR mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. Mol. Microbiol. 22, 535-544

Ferguson, A.D. et l. (1998) Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science 282(5397), 2215-2220

Fines, M. et al. (2001) Characterization of mutations in the *rpoB* gene associated with rifampin resistance in *Rhodococcus equi* isolated from foals. J. Clin. Microbiol. 39(8), 2784-2787

Fortier, A.H. et al. (1995) Growth of *Francisella tulerensis* LVS in macrophages: the acidic intracellular environment provides essential intracellular iron required for growth. Infection and Immunity 63, 1478–83.

Francis, J. et al. (1949) Isolation from Acid-Fast Bacteria of a Growth Factor for *Mycobacterium johnei* and a Precursor of Phthiocol. Nature 163, 365-366

Francis, J. et al. (1953) Mycobactin, a growth factor for *Mycobacterium johnei*. I. Isolation from *Mycobacterium pheli*. Biochem. J. 55, 596-607

Fuchs, H. et al. (1998) Structural model of phospholipid-reconstituted human transferrin receptor derived by electron microscopy. Structure 6, 1235–1243

Giguère, S. et al. (2010) Determination of the prevalence of antimicrobial resistance to macrolide antimicrobials or rifampin in *Rhodococcus equi* isolates and treatment outcome in foals infected with antimicrobial-resistant isolates of *R. equi*. JAVMA 237(1), 74-81.

Goetz, D.H. t al. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol. Cell. 10(5), 1033-1043

Gordeuk, V. et al. (1992) Effect of iron chelation therapy on recovery from deep coma in children with cerebral malaria. N. Engl. J. Med. 327, 1473-1477

Gordon, S. and Taylor, P.R. (2005) Monocyte and macrophage heterogeneity Nat. Rev. Immunol. 5(12), 953-64

Gruenheid, S. et al. (1999) The Iron Transport Protein NRAMP2 Is an Integral Membrane Glycoprotein That Colocalizes with Transferrin in Recycling Endosomes. JEM 189(5), 831-841

Guerinot, M. L. (1994) Microbial iron transport. Annu. Rev. Microbiol. 48, 743-772

Gwamaka, M. et al (2012) Iron deficiency protects against severe *Plasmodium falciparum* malaria and death in young children. Clin. Infect. Dis. 54(8), 1137-44

Halbert, N.D. et al. (2006) Variations in equid SLC11A1 (NRAMP1) genes and associations with *Rhodococcus equi* pneumonia in horses. J. Vet. Intern. Med. 20, 974–979

Harrington, J.R. et al. (2006) Antimicrobial activity of gallium against virulent *Rhodococcus equi* in vitro and in vivo. J. Vet. Pharmacol. Ther. 29, 121–127

Hershko, C. et al. (1992) The antimalarial effect of iron chelators: studies in animal models and in humans with mild falciparum malaria. J. Inorg. Biochem. 47, 267-277

Hentze MW, Muckenthaler MU, Galy B, et al. Two to tango: regulation of mammalian iron metabolism. Cell. 2010; 142:24–38.

Holmes, M.A. et al. (2005) Siderocalin (Lcn2) also binds carboxymycobactins, potentially defending against mycobacterial infections through iron sequestration. Structure 13, 29–41

Hondalus, M.K. and Mosser, D.M. (1994) Survival and replication of *Rhodococcus equi* in macrophages. Infect. Immun. 62, 4167–4175

Hubbard, J.A.M. et al. (1986) Effects of iron-limitation of *Escherichia coli* on growth, the respiratory chains and gallium uptake. Arch. Microbiol. 146, 80–86

Jordan, M.C. et al. (2003) Effects of iron modulation on growth and viability of *Rhodococcus equi* and expression of virulence-associated protein A. American Journal of Veterinary Research 64 (11), 1337-1346

Krewulak, K.D. and Vogel, H.J. (2008) Structural biology of bacterial iron uptake. Biochimica et Biophysica Acta 1778, 1781–1804

Thorstensen, K. and Romslo, I. (1990) The role of transferrin in the mechanism of cellular iron uptake Biochem J. 271(1), 1–9.

Krogstad, D.J. and Schlesinger, P.H. (1987) Acid-vesicle function, intracellular pathogens, and the action of chloroquine against *Plasmodium falciparum*. N. Engl. J. Med. 317(9). 542-549

Kunkle, C.A. and Schmitt, M.P. (2003) Analysis of the *Corynebacterium diphtheriae* DtxR regulon: Identification of a putative siderophore synthesis and transport system that is similar to the Yersinia high-pathogenicity island-encoded yersiniabactin synthesis and uptake system. J. Bacteriol. 185, 6826-6840

Letek, M. et al. (2010) The genome of a pathogenic Rhodococcus: cooptive virulence underpinned by key gene acquisitions. Plos Genet 6: e1001145.

Niwa, H. and Lasker, B.A. (2010) Mutant Selection Window and Characterization of Allelic Diversity for Ciprofloxacin-Resistant Mutants of *Rhodococcus equi*. Antim. Ag. Chemoter. 54 (8), 3520–3523

Ocampo-Sosa, A.A. et al (2007) Molecular epidemiology of *Rhodococcus equi* based on *traA*, *vapA*, and *vapB* virulence plasmid markers. J. Infect. Dis. 196, 763–769

Olakanmi, O. et al. (1994) Acquisition of iron bound to low molecular weight chelates by human monocyte-derived macrophages. J. Immunol. 153(6), 2691-703

Olakanmi, O. et al. (2000) Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. Infection and Immunity 68, 5619–5627

Masse, E. et al. (2007) Small RNAs controlling iron metabolism. Curr. Opin. Microbiol. 10, 140–145

Martens, R.J. et al. (2007) Chemoprophylactic antimicrobial activity of gallium maltolate against intracellular *Rhodococcus equi*. J. Equine Vet. Sci. 27, 341–345a

Martens, R.J. et al. (2007) Pharmacokinetics of gallium maltolate after intragastric administration in neonatal foals. Am. J. Vet. Res. 68, 1041–1044b.

Martens, R.J. et al. (2010) Gallium maltolate: Safety in neonatal foals following multiple enteral administrations. J. Vet. Pharmacol. Ther. 33, 208–212

McQueen, C.M. et al. (2015) Genetic Susceptibility to *Rhodococcus equi*. J. Vet. Intern. Med. 29 (6), 1648-1659

Miranda-CasoLuengo, R. et al. (2005) The iron-regulated iupABC operon is required for saprophytic growth of the intracellular pathogen *Rhodococcus equi* at low iron concentrations. J. Bacteriol. 187, 3438–3444

Miranda-CasoLuengo, R. et al. (2008) The intracellular pathogen *Rhodococcus equi* produces a catecholate siderophore required for saprophytic growth. J. Bacteriol. 190, 1631–1637

Mousel, M.R. et al. (2003) *Rhodococcus equi* and genetic susceptibility: assessing transferrin genotypes from paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation, 15, 470-472

Moller, M. and Hoal, E.G. (2010) Current findings, challenges and novel approaches to human genetic susceptibility to tuberculosis. Tuberculosis (Edinb) 90:71–83

Newton, J.R. et al. (2007) Evidence for transferrin allele as a host-level risk factor in naturally occurring equine respiratory disease: a preliminary study. Eq. Vet. J. 39 (2), 164-171

Nunez, M.T. et al. (1990) Mobilization of iron from endocytic vesicles - the effects of acidification and reduction. J. Biol. Chem. 265, 6688–6692

Ren, J. and Prescott, J.F. (2003) Analysis of virulence plasmid gene expression of intra-macrophage and in vitro grown *Rhodococcus equi* ATCC 33701. Vet. Microbiol. 94, 167-182

Raje, C.I. et al. (2007) The Macrophage Cell Surface Glyceraldehyde-3-phosphate Dehydrogenase Is a Novel Transferrin Receptor. J. Biological Chemist. 282 (5), 3252–3261

Ratledge, C. and Dover, L.G. (2000) Iron metabolism in pathogenic bacteria. Ann. Rev. Microbiol. 54, 881-941

Rodriguez, G.M. (2006) Control of iron metabolism in *Mycobacterium tuberculosis*. Trends Microbiol. 14, 320–327

Russell, D.G. (2001) *Mycobacterium tuberculosis*: here today, and here tomorrow. Nat Rev Mol Cell Biol. 2(8), 569-577

Saiga, H. et al. (2008) Lipocalin 2-dependent inhibition of mycobacterial growth in alveolar epithelium. J. Immunol. 81, 8521-8527

Sica and Mantovani, A. (2012) Macrophage plasticity and polarization: in vivo. J. Clin. Invest. 122(3), 787–795

Skamene, E. et al. (1982) Genetic regulation of resistance to intracellular pathogens. Nature 297(5866), 506–509

Smith, J.L. (2004) The Physiological Role of Ferritin-Like Compounds in Bacteria. Critical Reviews in Microbiology, 30, 173–185

Soe-Lin, S. et al. (2009) Nramp1 promotes efficient macrophage recycling of iron following erythrophagocytosis in vivo. Proc Natl Acad Sci USA 106, 5960–5965

Stintzi, A. et al. (2000) Microbial iron transport via a siderophore shuttle: A membrane ion transport paradigm Proc Natl Acad Sci USA 97(20), 10691–10696

Tao, X. et al. (1994) Iron, DtxR, and the regulation of diphtheria toxin expression. Mol. Microbiol. 14, 191-197

Tulliusa, M.V. et al. (2011) Discovery and characterization of a unique mycobacterial heme acquisition system. PNAS, 108 (12), 5051-5056

Venner, M. et al. (2012) Failure of antimicrobial therapy to accelerate spontaneous healing of subclinical pulmonary abscesses on a farm with endemic infections caused by *Rhodococcus equi*. Vet J. 192, 293–298

von Bargen, K. et al. (2011) Nitric Oxide-Mediated Intracellular Growth Restriction of Pathogenic *Rhodococcus equi* Can Be Prevented by Iron. Infection and immunity, 79 (5), 2098–2111

Yang, J. et al. (2002) An iron delivery pathway mediated by lipocalin. Mol. Cell. 10, 1045–1056

Richardson, D.R. (2005) 24p3 and its receptor: dawn of a new iron age? Cell. 123, 1175–1177

Wandersman, C. and Delepelaire, P. (2004) Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol, 58, 611–647

Watson, J.L. et al. (2002) Preliminary investigations of innate immunity to *Rhodococcus equi* infection. Dorothy Russell Havemeyer Foundation Workshop on *Rhodococcus equi* and equine rhodococcal pneumonia. Pullman, WA

Weinberg, G.A. (1994) Iron chelators as therapeutic agents against *Pneumocystis carinii*. Antimicrob Agents Chemother. 8(5), 997-1003

Weinberg, E.D. (1997) The Lactobacillus anomaly: total iron abstinence. Perspect. Biol. Med, 40, 578–583

Weiss G. Modification of iron regulation by the inflammatory response. Best Pract Res Clin Haematol 2005; 18: 183-201.

Wilks, A. (2002) Heme oxygenase: evolution, structure, and mechanism. Antioxid Redox Signal 4, 603–614

Wooldridge, K.G. and Williams, P.H. (1993) Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiol Rev. 12(4), 325-348

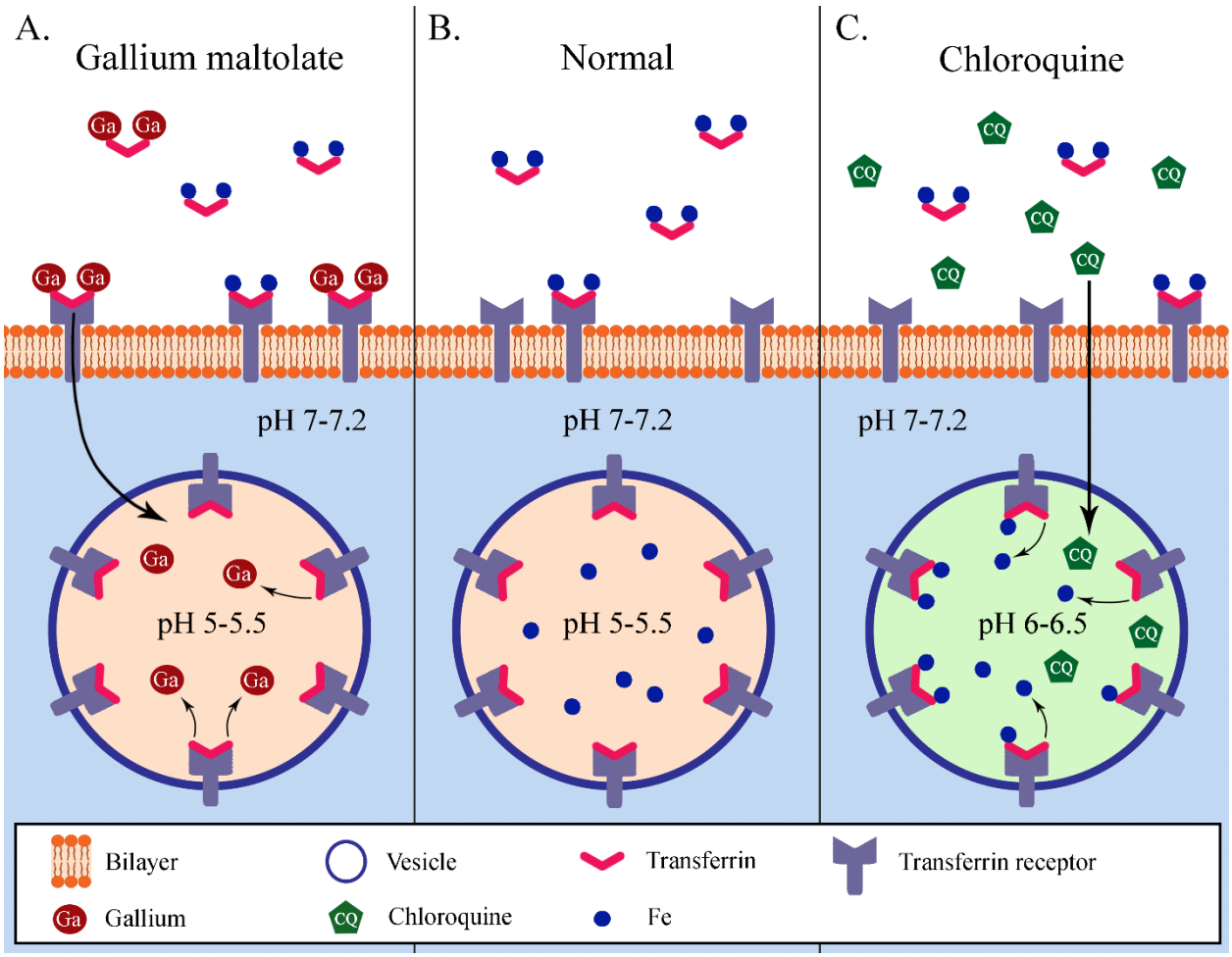

Figure 1. A) Macrophage plus Gallium maltolate: Ga acts as a "Trojan horse" modifying the Fe uptake and utilization by the microorganisms. In place of trivalent Fe, trivalent Ga binds to Tf and this complex is taken up through Tf receptors located on the macrophage cell surface. Following uptake, Ga is released inside acidic endosomal cytosolic vesicles and incorporated into the enzyme system of the intracellular pathogen. This errant incorporation leads to decreased enzyme function with consequent failure of bacteria to replicate and eventual death. B) Untreated macrophages: At a pH of 5.0 to 5.5, Tf becomes completely unsaturated and all Fe is free (unbound to Tf) within the vesicles. The unbound Fe can be used by the macrophage for normal metabolism, but also is available to intracellular pathogens like *R. equi*. Some of the Fe is incorporated and stored inside ferritin, some Fe remains available as a labile pool, and some is metabolized (for more details see section 5.). C) Macrophage plus Choroquine: Because CQ can raise the pH of the intracellular vesicles, some of the Fe cannot

be released from Tf (Tf requires a pH around 5.0 to 5.5 to become completely unsaturated). This markedly reduces availability of the intracellular (and intravesicular) Fe. Whereas macrophages can use stored Fe to counteract this decrease, intracellular pathogens must compete with the host cell for any available Fe. With continual exposure to CQ, even those pathogens able to produce siderophores, such as *R. equi*, will be inhibited or killed by Fe starvation, a mechanism which may be classified as a form of "nutritional immunity".

3. MANUSCRITO 2

Research paper

Chloroquine inhibits *Rhodococcus equi* replication in murine and foal alveolar macrophages by iron-starvation

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Chloroquine inhibits *Rhodococcus equi* replication in murine and foal alveolar macrophages by iron-starvation

Abstract

Rhodococcus equi preferentially infects macrophages causing pyogranulomatous pneumonia in young foals. Both the *vapA* and *rhbC* genes are up-regulated in an iron (Fe)-deprived environment, such as that found within macrophages. Chloroquine (CQ) is a drug widely used against malaria that suppresses the intracellular availability of Fe in eukaryotic cells. The main objective of this study was to evaluate the ability of CQ to inhibit replication of virulent *R. equi* within murine (J774A.1) and foal alveolar macrophages (AMs) and to verify whether the mechanism of inhibition could be Fe-deprivation-dependent. CQ effect on *R. equi* extracellular survival and toxicity to J774A.1 were evaluated. *R. equi* survival within J774A.1 and foal AMs was evaluated under CQ (10 and 20 μM), bovine saturated transferrin (bHTF), and bovine unsaturated transferrin (bATF) exposure. To explore the action mechanism of CQ, the superoxide anion production, the lysozyme activity, as well as the relative mRNA expression of *vapA* and *rhbC* were examined. CQ at ≤ 20 μM had no effect on *R. equi* extracellular multiplication and J774A.1 viability. Exposure to CQ significantly and markedly reduced survival of *R. equi* within J774A.1 and foal AMs. Treatment with bHTF did not reverse CQ effect on *R. equi*. Exposure to CQ did not affected superoxide anion production or lysozyme activity, however *vapA* and *rhbC* expression was significantly increased. Our results reinforce the hypothesis that intracellular availability of Fe is required for *R. equi* survival, and our initial hypothesis that CQ can limit replication of *R. equi* in J774A.1 and foal AMs, most likely by Fe starvation.

Keywords: Chloroquine, Iron, Macrophage, *rhbC*, *Rhodococcus equi*, *vapA*

1. Introduction

Rhodococcus equi is a Gram-positive, facultative intracellular coccobacillus that preferentially infects macrophages (Prescott, 1991). Chronic, abscessing bronchopneumonia caused by *R. equi* remains an important cause of disease and death in young foals (Prescott el al., 2010). Its survival and replication within macrophages depends on the presence of a virulence plasmid encoding 6 virulence-associated proteins, including VapA and the transcriptional regulators VirR and VirS, controlling the expression of *vapA* and required for remodeling the transcriptome to enhance survival of *R. equi* in the host intracellular environment (Hondalus and Mosser, 1994; Coulson et al., 2015). Expression of VapA is substantially up-regulated in an iron (Fe)-deprived environment, such as that found inside macrophages (Benoit et al., 2002; Jordan et al., 2003; Rahman et al., 2003; Ren and Prescott, 2003). Along with *vapA*, *rhbC* transcription is strongly up-regulated in *R. equi* during intra-macrophage infection. *rhbC* is part of the *rhbABCDE* gene cluster that encodes a hidroxamate siderophore (rhequichelin) required for intracellular growth and *in vivo* proliferation of *R. equi* (Miranda-CasoLuengo et al., 2012).

Adequate availability and metabolism of Fe is essential for many biological processes, such as respiration, DNA biosynthesis, and regulation of gene expression (Andrews et al., 2003). Consequently, Fe is an essential micronutrient for almost all bacteria including *R. equi* (Jordan et al., 2003; Rahman et al., 2003). Fe is released inside macrophages after acidification of vesicles containing Fe-bound-proteins such as transferrin (Tf) (Núñes et al., 1990; Andrews et al., 2003). To control replication of pathogens, mammalian cells lower Fe concentration as a mechanism of innate immunity referred to as “nutritional immunity” (Weinberg, 1975; Ratledge and Dover, 2000).

Chloroquine (CQ) is a weak base known to raise the pH of endocytic and lysosomal vesicles of eukaryotic cells (Krogstad and Schlesinger, 1987). This drug is widely used

against malaria and chronic inflammatory diseases, such that its therapeutic and safety properties are well-established in human medicine. By raising the vesicles' pH, CQ suppresses intracellular availability of Fe (Octave et al., 1979; Forsbeck and Nilsson, 1983; Swaiman and Machen, 1986; Baynes at al., 1987) which requires an acidic environment (Princiotto and Zapolski, 1975; Lestas, 1976; Byrd and Horwitz, 1989; Núñes et al., 1990; Byrd and Horwitz, 1991; Lane et al., 1991; Newman et al., 1994). Because of these proprieties, CQ has been found to possess strong activity against intracellular pathogens such as *Legionella pneumophila* (Byrd and Horwitz, 1991), *Histoplasma capsulatum* (Newman et al., 1994), *Francisella tularensis* (Fortier et al., 1995), and *Paracoccidioides brasiliense* (Dias-Melicio et al., 2006; 2007).

For decades, the combination of a macrolide with rifampin has been the standard of treatment of *R. equi* pneumonia in foals (Cisek et al., 2014). The emergence of macrolide and rifampin resistance (Giguère et al., 2010) and its association with mass antimicrobial treatment of foals to control *R. equi* pneumonia at affected farms (Burton et al., 2013) underscore the great need for alternative antimicrobials for treatment of *R. equi* pneumonia. Because the pathogenesis of *R. equi* is linked to intra-macrophage replication, because CQ can suppress the intracellular availability of Fe, and because of the indispensability of Fe for replication of *R. equi* replication, we examined whether CQ could inhibit intra-macrophage replication of virulent *R. equi* and whether the mechanism of inhibiting *R. equi* growth was Fe-deprivation-dependent. Results of this study would provide evidence about the potential use of CQ as an alternative treatment for rhodococcal pneumonia in foals.

2. Material and methods

2.1 Bacterium and inoculum preparation

For all experiments described herein, the inoculum was standardized from a single colony of *R. equi* ATCC 33701P+, *vapA*-positive, grown in brain heart infusion broth (BHIB; Beckton-Dickinson, Cockeysville, MD, USA) for 24 h at 35 ºC with rotation (10 revolutions per minute - r.p.m; Laboratory rotator, model 099A; Glas-Col, Terre Haute, IN, USA). Bacterial cells were pelleted by centrifugation at 4000 r.p.m. for 10 min and washed 2 times with sterile phosphate-buffered saline (PBS; Gibco BRL, Frederick, MD, USA). The concentration of bacteria was determined spectrophotometrically (Synergy 2 Multi-Mode Reader, BioTek) at an optical density of 600 nm (OD 600), where OD 1.0 represent approximately 2 x 10^8 CFU/ml. Several aliquots of *R. equi* suspended in PBS were stored frozen at -80 °C until used in the experiments described below.

2.2 Effects of CQ against R. equi extracellularly

The minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively) of CQ against *R. equi* were assessed in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). *Rhodococcus equi* (1 x 10^5 CFU) were inoculated into 96-well microtiter plates containing 19.5 µM to 40 mM CQ. Erythromycin was used for quality control testing because *R. equi* ATCC® 3370™ is known to be sensitive to it (Giguère et al., 2010). The MIC was defined as the lowest concentration of CQ that inhibited the visible growth of *R. equi* (media turbidity) after incubation for 24 h at 35°C. The MBC was defined as the lowest concentration of CQ that inhibited the visible growth of *R. equi* (CFU) by plating 10 µl from each well on Mueller-Hinton agar (MHA) following incubation for 24 h at 35°C. This experiment was performed in triplicate.

2.3 Chloroquine toxicity to murine macrophages J774A.1

The viability of J774A.1 macrophages exposed to CQ at different concentrations was evaluated using flow cytometry. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and 5% nonessential amino acids. The cells were harvested and resuspended at a concentration of 1 x 10^6 cells/ml, and 1 ml of cell suspension was placed in each well of 24-well tissue culture plates and incubated (37 °C, 5% CO_2) for at least 3 h to allow adherence. Then, the media were removed and replaced with media containing 0, 5, 10, 20, 50, 100, 200, 400, and 800 µM CQ. The cells were incubated at 37 °C with 5% CO_2 for 24 h, then placed on ice for 15 min, followed by pipetting up and down 5 to 10 times to detach cells from the plates. The cells were transferred to 1.5 ml tubes and centrifuged at 300 g for 5 min. The cell-pellet was resuspended in Alexa Fluor® 647 succinimidyl ester (Life Technologies, Carlsbad, CA, USA). Cells were fixed with 1% paraformaldehyde (Sigma, St. Louis, MO, USA). The proportions of dead and live cells (positive and negative populations, respectively) were determined using a flow cytometer (Attune NxT Flow Cytometer, Life Technologies, USA).

2.4 Effects of CQ on phagocytosis of R. equi by J774A.1 ability

Murine macrophages (J774A.1) were maintained as described in section 2.2 and resuspended at a concentration of 1 x 10^5 cells/ml, and 1 ml of cell suspension was placed in each well of 24-well tissue culture plates and incubated (37 °C, 5% CO_2) for at least 3 h to allow adherence. The media were then replaced with 0, 10, 20, or 50 µM CQ, and the cells were incubated (37 °C, 5% CO_2) for 0.5, 1, 2, or 4 h. At each time, the media were removed, the monolayers were washed once with PBS, and 1 ml of media containing 1 x 10^6 (i.e., multiplicity of infection [MOI] 10) *R. equi* was added to each well for 30 min to allow for phagocytosis. The media were then removed, monolayers were washed 2 times with warm

PBS, and the cells were lysed by adding 1 ml of sterile water followed by 45 min of incubation at 37 °C. Individual wells were scraped with the tip of a pipet before the contents were transferred to a 1.5 ml tube. The lysates were vortexed for 5 min, followed by 5 min of sonication, and by an additional 5 min of vigorous vortexing. Ten-fold serial dilutions of the lysate were plated onto BHI plates and the number of *R. equi* CFU was determined after 48 h incubation at 35 °C. Untreated and uninfected as well as untreated and infected macrophage monolayers cultured under the same conditions were used as controls. The results of the triplicates wells were used for data analysis.

2.5 R. equi survival in J774A.1 macrophages exposed to CQ treatment

To determine the effect of CQ on *R. equi* viability during intracellular infection, the J774A.1 cells were prepared as described before, until the adherence step. The cells were then exposed to 0, 5, 10, or 20 µM CQ for 24 h either before or after infection with *R. equi* (N.B., the cells were *not* continuously exposed to CQ). Besides CQ treatment, we evaluated the influence of Tf proteins by pre-incubation of the cells for 24 h before infection with the following treatments: 1) CQ (10 or 20 µM) plus bovine holotransferrin (bHTF; 99% iron-saturated; 6 mg/ml); 2) CQ (10 or 20 µM) plus bovine apotransferrin (bATF; 0% iron-saturated; 6 mg/ml); 3) bHTF only; and, 4) bATF only. To infect the macrophages, media were removed, the monolayers were washed once with PBS, and 1 ml of media containing 1 x 10^6 (MOI 10) *R. equi* was added to each well for 30 min to allow phagocytosis. The cells were then washed 2 times with PBS to remove extracellular organisms and supplemented with gentamicin at 8 µg/ml. At specific times after infection, the number of intracellular organisms was determined by viable counts on agar culture plates. The number of CFU within macrophages was determined at 0 (30 min after infection), 24, 48, and 72 h post-infection at 37 °C in 6% CO_2. At a given time-point, the viable *R. equi* were recovered as described

above. Untreated and uninfected as well as untreated and infected macrophage monolayers cultured under the same conditions were used as controls. The mean of triplicate wells was used for data analysis and the results were expressed as the reduction in the number of *R. equi* CFU relative to the infected untreated monolayers.

2.6 R. equi survival in foal AMs exposed to CQ

Bronchoalveolar lavage (BAL) fluid was collected from 5 healthy Quarter Horse foals (ages 2 to 28 days) as described previously (Burton e al., 2015). Bronchoalveolar cells were washed 3 times with PBS, counted, and suspended at a concentration of 1 x 10^6 cells/ml in minimum essential medium-alpha (αMEM, Lonza, Cat. N. BE02-002F) containing 10% heat-inactivated horse serum and amphotericin B (25 µg/ml). One ml of this suspension was added to each well of a 24-well plate (Nunc, ThermoFisher Scientific, Rochester, NY) and incubated for 24 h at 37 °C in 6% CO_2. Bronchoalveolar lavage cells were washed twice with warm media to remove non-adherent cells and then incubated with either 0, 10 or 20 µM CQ for 24 h at 37 °C in 6% CO_2. The cells were then washed and infected with *R. equi* (MOI 10) in αMEM supplemented with non-heated-inactivated 10% horse serum. Uninfected cells cultured under the same conditions were used as negative controls. Cells were then incubated for 45 min at 37 °C in 5% CO_2 to allow phagocytosis; the number of CFU within foal AMs was determined at 0 h (45 min after infection) and 48 h post-infection as described above. For each foal, the mean of duplicate wells was used for data analysis. Results were expressed as the reduction in the number of *R. equi* CFU (log_{10}/well) relative to the untreated control monolayers of each horse.

2.7 Effect of CQ on superoxide anion production and lysozyme activity by J774A.1 macrophages

To determine both superoxide anion production and lysozyme activity, J774A.1 macrophages were infected as described in section 2.2. The macrophages were exposed to either 0, 10, or 20 μM CQ during 24 h prior to being infected. The cell-free supernatants were recovered from untreated and uninfected cells, untreated and infected cells, and treated and *R. equi*-infected cells (MOI 10) at 12 and 24 h post-infection. Supernatants from t 0 h (i.e., just macrophages in the presence or absence of CQ) were also recovered. The following tests were performed using 3 biological replicates and 2 technical replicates.

2.7.1 Assay for determination of superoxide anion production

Because intracellular killing is also dependent on oxidants, superoxide anion production has been associated with many physiological and pathological processes including host innate immune and inflammatory responses to pathogens. Thus, we determined the amount of superoxide anion produced and released by macrophages exposed to CQ. The superoxide anion production assay measures the change in color of cytochrome C (cyt C) when reduced by oxygen. This method was slightly modified from Dey and Bishayi (2015). The collected supernatants (100 μL) were incubated in the presence of cyt C (100 μL at 2 mg/mL in HBSS) and thereafter the production of superoxide anion was monitored spectrophotometrically at 550 nm (Synergy 2 Multi-Mode Reader, BioTek). Based on the extinction coefficient of reduced–oxidized cyt C (OD = 21×10^3 $M^{-1}cm^{-1}$) and because the vertical light path was 3 mm, the absorbance value could be converted to nmol of superoxide anion based on the following formula: nmol of superoxide anion = (mean absorbance at 550 nm × 15.87). Results were expressed in nmol/10^6 macrophages.

2.7.2 Assay for determination of lysozyme activity

Lysosomal enzymes are necessary to release additional ligands and enhance innate signaling, especially against intracellular pathogens capable of inhibiting lysosomal fusion and/or acidification. We therefore estimated lysozyme enzyme activity by macrophages exposed to CQ. The same supernatants used to determine the amount of superoxide anion production were used to estimate the lysozyme activity, according to the method described by Colowick et al. (1986), with minor modifications. A suspension of 40 mg of *Micrococcus lysodeikticus* (Sigma, M0508) in 200 ml of 0.15 M potassium phosphate buffer at pH 6.2 was used. Ten µL of supernatant were added to each well and the reaction was started by adding 200 µL of *M. lysodeikticus* suspension. The decrease in OD was recorded at 450 nm (Synergy 2 Multi-Mode Reader, BioTek) as a function of time. One unit of enzyme was defined as the amount of enzyme produced a decrease in absorbance of 0.001/min at 450 nm.

2.8 Transcription of vapA and rhbC by R. equi intracellularly: J774A.1 cells pretreated with CQ, HTF and APO

2.8.1 Macrophage infection

For intra-macrophage gene expression analysis, J774A.1 cells were seeded at 6 x 10^5 cells/ml in a 6-cm tissue culture plate and cultured overnight at 37°C with 5% CO_2. The cells were exposed to 10 or 20 µM CQ only, 20 µM CQ plus bHTF (6 mg/ml), bHTF (6 mg/ml) only, or bATF (6 mg/ml) only for 24 h prior to infection. *Rhodococcus equi* grown in BHI broth were harvested by centrifugation (10 min at 3,220 x g) in the exponential phase of growth and were washed 2 times with PBS. Then J774A.1 cells were infected with *R. equi* at an MOI of 20 as previously described (Miranda-CasoLuengo et al., 2012). Briefly, macrophages were infected by centrifugation (3 min at 160 g) of bacteria onto confluent macrophage monolayers to synchronize internalization. After 30 min of incubation to allow

phagocytosis, the cells were washed 2 times with PBS to remove extracellular organisms and supplemented with gentamicin (8 µg/ml). The first samples were harvested 1 h after addition of medium supplemented with gentamicin, which was considered t 0 h. Infected monolayers were also harvested at 12 h post-infection.

2.8.2 RNA isolation

Rhodococcus equi RNA was isolated from macrophages at 0 h and 12 h post-infection using a guanidine thiocyanate-based lysis buffer (supplied by Qiagen RNase Kit, Cat. No. 74104) as previously described (Miranda-CasoLuengo et al., 2012). Samples were vortexed and passed 10 times through a 21-gauge needle to shear macrophage DNA and to reduce viscosity. Intracellular bacteria were recovered by 30 min of centrifugation at 3220 g. Pelleted bacteria were lysed using TRIzol (Sigma, Cat. No. T9424) and physically disrupted with 0.01-mm zirconia beads. Total RNA was isolated by chloroform extraction followed by application to a Qiagen RNeasy column to perform an in-column DNA digestion with the RNase-free DNase Set (Qiagen, Cat. No. 79254) as described by the manufacturers.

2.8.3 Reverse transcription, qPCR and data analysis

cDNA was transcribed by extension of hexameric random primers with SuperScript® III First-Strand Synthesis System (Invitrogen™, Cat. No. 18080-051) following the manufacturer's directions. DNA contamination of RNA samples was ruled out by including controls with no addition of reverse transcriptase. Quantitative PCR (qPCR) using appropriate oligonucleotides (Table 1) from TaqMan probe (Applied Biosystems, Life Technologies, Grand Island, NY, USA) were performed in optical 384-well plates in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the TaqMan Universal Master Mix (Invitrogen, Life Technologies, Grand Island, NY, USA). The thermal profile

consisted of an initial hold at 50 °C for 2 min, followed by a single denaturation step at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The resulting threshold cycle (Ct) data for all wells were exported to RQ Study software Applied Biosystems for further analysis. The software uses the comparative method ($2^{-\Delta\Delta Ct}$) of relative quantification (for more information refer to *ABI PRISM 7700 Sequence Detection System User Bulletin #2* – PN 4303859). At least 3 independent experiments were performed in duplicate for each sample, which represented duplicates for each qPCR reaction. Fold changes in transcript level were normalized to that of the *16S* rRNA and *gyr* gene for the qPCR reactions.

2.9 Statistical analysis

Phagocytosis data were analyzed using linear mixed-effects modeling to account for effects of time, concentration of chloroquine, and their interaction on the outcome of the CFU of *R. equi*. To better fit model assumptions, the CFU data were converted using the $\log_{10}$ function for analysis. To evaluate the effects of treatments on infected murine macrophages or foal AMs, data were analyzed using linear mixed-effects modeling where the outcome was the reduction of CFU of *R. equi* ($\log_{10}$-transformed) relative to the untreated control, treatment was modeled as a fixed categorical effect, and experiment (for murine macrophages) or foal (for AMs) was modeled as a random effect. Lysozyme and cytochrome data were analyzed using linear mixed-effects modeling to account for effects of replicates nested within experiment (as a random effect) and fixed effects of time, concentration of CQ, *R. equi*, and their interactions, and using either lysozyme activity or superoxide anion release as the outcomes of interest. The qPCR data were analyzed using linear mixed-effects regression with the RQ of a given gene as the outcome, treatments were modeled as a fixed, categorical effects, and replicate nested within experiment was modeled as a random effect. Post-hoc testing of significant differences among treatments were made using the method of Sidak. For

the GLM and linear-mixed effect modeling, model fit was assessed using diagnostic residual plots. Significance was set at $P < 0.05$. Analysis was performed using S-PLUS (version 8.2, TIBCO, Inc., Seattle, WA).

3. Results

3.1 Chloroquine toxicity to R. equi extracellularly and murine macrophages J774A.1

The MIC of CQ against *R. equi* was 1.25 mM, while the MBC was > 40 mM. Using the flow cytometry method, we verified that J774A.1 macrophages exposed to concentrations of CQ ranging from 5 to 50 µM showed viability similar to the untreated cells. CQ concentrations between 100 to 800 µM, however, significantly decreased viability of these macrophages.

3.2 Effect of CQ on the phagocytic capacity of J774A.1 macrophages for R. equi

For a given CQ concentration, there were no significant differences in phagocytic capacity among the 4 times after time 0 (Table 2). At each time-point, however, J774A.1 cells exposed to 50 µM CQ showed significantly lower phagocytic capacity for *R. equi* than cells exposed to all other CQ concentrations, including the negative control (i.e., 0 µM CQ).

3.3 R. equi viability in J774A.1 macrophages exposed to CQ and Tf proteins

3.3.1 CQ reduces R. equi viability in J774A.1 macrophages

There were significant differences among treatment groups after 24, 48, and 72 h (Fig. 2). At 24 h, both 5 µM CQ treatments (before and after infection) were not significantly different from the control treatment (i.e., 0 µM CQ). Concentrations of 10 and 20 µM CQ, however, caused a significant reduction of viable *R. equi* compared to the control treatment. The same results were observed at 48 h. After 72 h, in addition to the 5 µM treatment, the 10 µM CQ

treatment pre-infection also was not significantly different than the control treatment, whereas the other treatments resulted in significant reduction of viable *R. equi* compared to control group.

3.3.2 bHTF does not reverse CQ effect on R. equi viability in J774A.1 macrophages

There were significant differences among treatment groups 48 h post-infection (Fig. 3). Treatments of bHTF + 10 µM CQ (P = 0.0037), bHTF + 20 µM CQ (P = 0.0009), bATF + 10 µM CQ (P = 0.0128), and bATF + 20 µM CQ (P = 0.0015) strongly inhibited *R. equi* growth. Although bHTF is known to supply Fe to enable survival of *R. equi* (Jordan et al., 2003) the cells treated with bHTF (6 mg/ml) + CQ (20 µM) did not significantly (P = 0.4407) reverse the inhibition of *R. equi* growth, as expected from the ability of CQ to prevent Fe release from Tf (Krogstad and Schlesinger, 1987). To control for the effects of the bHTF treatment, macrophages were cultured under the same conditions with CQ + bATF (6 mg/ml) in the place of bHTF, or bATF only (6 mg/ml). Interestingly, bATF showed a modest but significant (P = 0.0421) inhibition of *R. equi* viability (Fig. 3).

3.4 CQ reduces R. equi viability in foal AM

There were significant differences among treatment groups at 48 h post-infection (Fig. 4). Both 10 µM CQ (P = 0.0012) and 20 µM CQ (P = 0.0002) significantly inhibited *R. equi* multiplication in foal AMs under the conditions analyzed.

3.5 Effect of CQ on superoxide anion production and lysozyme activity by J774A.1 macrophages

3.5.1 Superoxide anion production

There were significant ($P < 0.05$) effects on superoxide anion production under time that depended on CQ treatment (Table 3), but not on *R. equi* presence ($P = 0.1982$). In the absence of CQ there were no significant differences in the amount of superoxide anion (nmol/10^6 cells) in the supernatant at 12 h ($P = 0.8050$) or 24 h ($P = 0.4976$) relative to time 0 h. At a concentration of 10 µM CQ, the amount of superoxide anion at 12 h was significantly ($P = 0.0001$) greater than at time 0 h; however, values for 10 µM CQ were not significantly ($P = 0.10083$) greater at 24 h than 0 h. At a concentration of 20 µM CQ, there were no significant differences among the 3 times in the amount of superoxide anion. Values of superoxide anion were significantly ($P = 0.0399$) lower at time 0 h for CQ concentration of 10 µM than concentration 0 µM; however, there was no significant difference between 10 and 20 µM CQ at time 0 h. No other differences among times or CQ concentrations were significant.

3.5.2 Lysozyme activity

There were significant ($P < 0.05$) effects on lysozyme activity (Table 4) of time and CQ treatment, but not of *R. equi* presence ($P = 0.6797$). For a given time, the lysozyme activity values were significantly lower for 10 ($P = 0.0124$) and 20 µM CQ ($P = 0.0015$) than for untreated (control) macrophages. For a given concentration of CQ, lysozyme activity was significantly ($P < 0.0001$) lower at 12 h than at time 0 h, and significantly ($P < 0.0001$) higher at 24 h than time 0 h (or time 12 h).

3.6 Transcription of vapA and rhbC in R. equi intramacrophage

To confirm the assumption that CQ generated an Fe-deficient condition, we analyzed the relative expression of *vapA* and *rhbC* in *R. equi* after macrophage infection. The results of relative *vapA* expression were represented graphically (Fig. 5). At time 0 h (1 h after the media change), *vapA* transcription was significantly ($P < 0.05$) greater in *R. equi* exposed to

either 10 or 20 µM CQ treated cells than untreated cells. Treatment with bATH significantly ($P < 0.05$) increased *vapA* expression, whereas bHTF significantly ($P < 0.05$) decreased relative *vapA* expression even in the presence of 20 µM CQ. At time 12 h, there were significant effects of 20 µM CQ and bHTF, but not the other treatments (10 µM CQ, 20 µM CQ plus bHTF, or bATF).

The results of relative *rhbC* expression were represented graphically (Fig. 6). At time 0 h, the expression of *rhbC* was significantly ($P < 0.05$) greater by *R. equi* exposed to either 10 µM or 20 µM CQ-treated cells than by untreated *R. equi*. Treatment with bATF significantly ($P < 0.05$) increased *rhbC* expression by *R. equi*, whereas HTF significantly ($P < 0.05$) decreased its expression even in the presence of 20 µM CQ. At 12 h, there were significant effects of 10 µM and 20 µM CQ (*rhbC* up-regulation), 20 µM CQ plus bHTF, and bHTF (*rhbC* down-regulation), but not the bATF treatment.

4. Discussion

Chloroquine inhibits the growth of several intracellular microorganisms for which Fe is essential for survival. Because CQ suppresses intracellular availability of Fe, we evaluated whether CQ could inhibit *R. equi* replication within macrophages by an Fe-deprivation-dependent mechanism. Here we show that CQ can prevent *R. equi* replication in murine macrophages (J77A.1) and foal AMs, most likely by Fe starvation.

We first determined the MIC of CQ for *R. equi* in culture media. Replication of *R. equi* was inhibited at 1.25 mM CQ, similar to the concentration observed to inhibit growth of *M. tuberculosis* (1 mM) (Crowle et al., 1990). The inhibitory effect of CQ appears to be due its intercalation into bacterial DNA at concentrations around 1 mM (Ciak and Hahn, 1966; Crowle et al., 1986). Although CQ inhibited replication of *R. equi* at 1.25 mM, the bacterium was able to grow even when exposed to concentrations of CQ up to 40 mM. *Mycobacterium*

avium (Crowle et al., 1986) and *M. tuberculosis* (Crowle et al., 1990) appear to have the same tolerance to CQ because they remained viable at a CQ concentration of 16 mM. Thus, we concluded that the effect of CQ at concentrations ≤ 20 μM on growth of *R. equi* would not be attributable to direct damage of CQ to the bacterium.

To document that the observed effects of CQ were not attributable to toxicity to macrophages, we evaluated the viability of J774A.1 cells exposed to CQ at different concentrations using flow cytometry. We found that CQ up to 50 μM was not toxic to J774A.1 cells; reduced viability of these macrophages was observed at concentrations from 100 to 800 μM CQ. Similarly, Jones and Jayson (1984) reported that concentrations of 100 μM CQ had marked deleterious effects on human neutrophils but exposure to 10 μM did not; Yoon et al. (2010) observed that CQ concentrations ≥ 100 μM induced vacuole formation and death of epithelium-derived cells. On the basis of prior reports and our findings, we conclude that concentrations of 20 μM or less do not cause toxicity to J774A.1 macrophages.

We also examined the effects of CQ on the ability of J774A.1 macrophages to phagocytose

R. equi. A time- and dose-dependent effect was observed, as has been previously reported (Sung et al., 1983; Jones and Jayson, 1984). In the present study, 10 and 20 μM CQ did not cause significant effects on *R. equi* phagocytosis compared to untreated control macrophages; however, 50 μM CQ significantly ($P < 0.05$) inhibited phagocytosis of *R. equi* in these cells (Table 2). Sung et al. (1983) observed 20% and 25% reduction of phagocytosis after 1h of pre-incubation with 10 and 50 μM CQ, respectively. Although neither we nor Levitz et al. (1997) observed reduction of phagocytosis of bacteria at 10 μM, we did observe approximately 20% inhibition after 1 h of pre-incubation with 50 μM CQ. The exact mechanism by which CQ decreases phagocytosis is yet unknown, but down-regulation of macrophage phagocytosis receptors (Tietze et al., 1980) and inhibition of production of new

plasma membrane (required for the accommodation of phagocytized particles; Matsusawa and Hostetler, 1980) have been suggested as possible causes.

Fe is an essential micronutrient for *R. equi* multiplication, both in its saprophytic lifestyle as well as inside macrophages where there is an environment of Fe restriction (Jordan et al., 2003; Miranda-CasoLuengo et al., 2012). We observed strong inhibition of *R. equi* multiplication inside J774A.1 macrophages and foal AMs exposed to CQ (Fig. 1 and 3). In addition, our hypothesis that CQ acts by a mechanism that is Fe-deprivation-dependent was supported by the results in which bHTF did not reverse the effects of CQ on inhibiting replication of *R. equi* (Fig. 2). As reported previously, HTF supplies Fe to *R. equi* thereby enabling the bacteria to survive (Jordan et al., 2003; Miranda-CasoLuengo et al., 2012); however, in macrophages the vesicle must be acidified to a pH of approximately 5.0 to 6.0 for 100% of ferric iron to be dissociated from HTF and released it into the cytoplasm (Princiotto and Zapolski, 1975; Núñez et al., 1990). Thus, by increasing the pH of lysosomal and endocytic vesicles, CQ has been found to markedly reduce the availability of Fe in several cell types, including mononuclear phagocytes and macrophages (Octave et al., 1979; Forsbeck and Nilsson, 1983; Swaiman and Machen, 1986; Baynes et al., 1987; Dias-Melicio et al., 2007). Alternatively, CQ might block release of Fe from ferritin, a macrophage storage compound for Fe (Andrews et al., 2003), which should be degraded in lysosomes (when acidified) and able to release a pool of Fe intracellularly (Sibille et al., 1989).

To confirm the assumption that CQ generated an Fe-deficient condition, we analyzed the relative expression of *vapA* and *rhbC* in *R. equi* after macrophage infection. Both genes are strongly up-regulated in an Fe-deprived environment, such as found within macrophages (Benoit et al., 2002; Ren and Prescott, 2003; Miranda-CasoLuengo et al., 2012). J774A.1 macrophages were pretreated for 24 h with 10 or 20 µM CQ, 20 µM CQ + bHTF, bHTF only, and bATF only, and subsequently infected with *R. equi* (MOI 20). The *R. equi* were harvested

at time 0 h (i.e., 1 h after the media changing post-infection) and 12 h post-infection. As expected, CQ treatment significantly increased mRNA expression of *vapA* and *rhbC* ($P < 0.05$). We also observed significantly increased mRNA expression of these genes during bATF treatment (Fig. 4 and 5). As for CQ, bATF showed modest but significant ($P = 0.0421$) inhibition of *R. equi* replication in J774A.1 macrophages 48 h post-infection (Fig. 2). Although it is difficult to predict the ATF amount required to inhibit microbial growth due to the complex dynamic of free Fe versus bound Fe, ATF is considered a biological method for inducing Fe starvation (Lin et al., 2014).

In contrast to bATF, bHTF + CQ and bHTF alone reduced the relative expression of *vapA* and *rhbC* ($P < 0.05$), especially on samples harvested at 0 h (Fig. 4 and 5). Considering that bHTF is an important source of Fe for macrophages (and, consequently, for *R. equi*), we expected to find down-regulation of *vapA* and *rhbC* expression relative to untreated controls, which were intended to represent the "basal" Fe-restricted environment encountered within macrophages. However, bHTF + CQ treatment also caused down-regulation of *vapA* and *rhbC* expression compared to control ($P < 0.05$). It is important to note that although CQ raises the pH to around 6.5 (Núñez et al., 1990; Newman et al., 1994), release of Fe from HTF is not completely inhibited at this pH: approximately 50% of Fe is dissociated from HTF at pH 6.5, and the remainder is released only at pH between 5.0 and 6.0 (Princiotto and Zapolski, 1975; Núñez et al., 1990). Thus, the additional HTF might have created a counterbalance to the CQ inhibition of Fe release, thereby making adequate Fe available to inhibit the induction of mRNA expression of *vapA* and *rhbC*.

Enhanced phagolysosome formation, induction of autophagy, and production of toxic radicals, such as nitric oxide (NO), have been related to inhibition of intracellular replication of mycobacterial organisms (Sibille et al., 1989; Schaible et al., 1998; Via et al., 1998). Conversely, inhibition of replication of *R. equi* in activated macrophages does not appear to

be attributable to autophagy, and NO-mediated killing of *R. equi* can be prevented by Fe excess (von Bargen et al., 2011). We found no significant increase in superoxide anion production or lysozyme activity by macrophages exposed to CQ (Tables 3 and 4). Some reports indicate that the activity of CQ is not attributable to induction of bactericidal compounds such as superoxide anions and lysozyme (Dias-Melicio et al., 2007; Day and Bishayi, 2015), but rather is caused by Fe restriction. Thus, CQ might represent an alternative to *R. equi* growth restriction, even in resting macrophages.

5. Conclusion

Our results reinforce the hypothesis that availability of Fe is required for intracellular survival of *R. equi* (Jordan et al., 2003; Miranda-CasoLuengo et al., 2012). We demonstrate that CQ is capable of killing *R. equi* in J774A.1 murine macrophages and foal AMs, most likely by Fe deprivation. Moreover, the potential exists for using CQ as a tool for investigating the intracellular trafficking and Fe acquisition of *R. equi*. Additional *in vivo* studies are needed to assess the pharmacokinetics, pulmonary disposition, safety, and therapeutic potential of CQ as a treatment for *R. equi* infection of foals.

Conflicts of interest: none

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References

Andrews, S.C., Robinson, A.K., Rodriguez-Quinones, F., 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. 27, 215–237.

Baynes, R., Bukofzer, G., Bothwell, T., Bezwoda, W., Macfarlane, B., 1987. Transferrin receptors and transferrin iron uptake by cultured human blood monocytes. Eur. J. Cell. Biol. 43:372-376.

Benoit, S., Benachour, A., Taouji, S., Auffray, Y., Hartke, A., 2002. H_2O_2, which causes macrophage-related stress, triggers induction of expression of virulence-associated plasmid determinants in *Rhodococcus equi*. Infect. Immun. 70, 3768–3776.

Burton, A.J., Giguère, S., Berghaus, L.J., Hondalus, M.K., Arnold, R.D., 2015. Efficacy of liposomal gentamicin against *Rhodococcus equi* in a mouse infection model and colocalization with *R. equi* in equine alveolar macrophages. Vet. Microbiol. *176(3), 292-300.*

Burton, A.J., Giguère, S., Sturgill, T.L., Berghaus, L.J., Slovis, N.M., Whitman, J.L., Levering, C., Kuskie, K.R., Cohen, N.D., 2013. Macrolide and rifampin resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA. Emerg. Infect. Dis. 19, 282–285.

Byrd, T.F., Horwitz, M.A., 1989. Interferon-gamma activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. J. Clin. Invest. 83, 1457–1465.

Byrd, T.F., Horwitz, M.A., 1991. Chloroquine inhibits the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. A potential new mechanism for

the therapeutic effect of chloroquine against intracellular pathogens. J. Clin. Invest. 88, 351–357.

Ciak, J., Hahn, F.E., 1966. Chloroquine: mode of action. Science. 151(3708), 347-349.

Cisek, A.A., Rzewuska, M., Witkowski, L., Binek, M., 2014. Antimicrobial resistance in *Rhodococcus equi*. Acta. Biochim. Pol. 61(4), 633-638.

CLSI (Clinical and Laboratory Standards Institute)., 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals (CLSI document VET01-A4). Approved Standard. 4th ed. Waye, PE.

Colowick, S.P., Kaplan, N.O., Absolom, D.R., 1986. Basic methods for the study of phagocytosis. In: Sabato, G,D,, Everse, J, (Eds), Methods in Enzymology. Immunochemical techniques part J: phagocytosis and cell-mediated cytotoxicity. Academic Press, New York, NY

Coulson, G.B., Miranda-CasoLuengo, A.A., Miranda-CasoLuengo, R., Wang, X., Oliver, J., Willingham-Lane, J.M., Meijer, W.G., Hondalus, M.K., 2015. Transcriptome reprogramming by plasmid encoded transcriptional regulators is required for host-niche adaptation of a macrophage pathogen. Infect. Immun. 83, 3137-3145.

Crowle, A.J., May, M.H., 1990.Inhibition of tubercle bacilli in cultured human macrophages by chloroquine used alone and in combination with Streptomycin, Isoniazid, Pyrazinamide, and two metabolites of vitamin D3. Antimicrob. Agents Chemother. 34(11), 2217-2222.

Crowle, A.J., Tsang, A.Y., Vatter, A.E., May, M.H., 1986. Comparison of 15 laboratory and patient-derived strains of *Mycobacterium avium* for ability to infect and multiply in cultured human macrophages. Infect. Immun. 24, 812-821.

Day, S., Bishayi, B., 2015. Killing of *Staphylococcus aureus* in murine macrophages by chloroquine used alone and in combination with ciproflxacin or azithromycin. J. Inflmm. Res. 8, 29-47.

Dias-Melicio, L.A., Calvi, S.A., Bordon, A.P., Golim, M.A., Peraçoli, M.T.S., Soares, A.M.V.C., 2007. Chloroquine is therapeutic in murine experimentalmodel of paracoccidioidomycosis. FEMS Immunol. Med. Microbiol. 50, 133–143.

Dias-Melicio, L.A., Moreira. A.P., Calvi. S.A., de Campos Soares, A.M.V., 2006. Chloroquine inhibits *Paracoccidioides brasiliensis* survival within human monocytes by limiting the availability of intracellular iron. Microbiol. Immunol. 50(4), 307–314.

Forsbeck, K., Nilsson, N., 1983. Iron metabolism of established human hematopoietic cell lines in vitro. Exp. Cell Res 14:323–332.

Fortier, A.H., Leiby, D.A., Narayanan, R.B., Asafoadjei, E., Crawford, R.M., Nacy, C.A., Meltzer, M.S., 1995. Growth of *Francisella tulerensis* LVS in macrophages: the acidic intracellular environment provides essential intracellular iron required for growth. Infect. Immun. 63, 1478–83.

Giguère, S., Lee, E., Williams, E., Cohen, N.D., Chaffin, M.K., Halbert, N., Martens, R.J., Franklin, R.P., Clark, C.C., Slovis, N.M., 2010. Determination of the prevalence of antimicrobial resistance to macrolide antimicrobials or rifampin in *Rhodococcus equi* isolates and treatment outcome in foals infected with antimicrobial-resistant isolates of *R. equi*. J. Am. Vet. Med. Assoc. 237(1), 74-81.

Harrington, J.R., Golding, M.C., Martens, R.J., Halbert, N.D., Cohen, N.D. 2005. Evaluation of a real-time quantitative polymerase chain reaction assay for detection and quantitation of virulent *Rhodococcus equi*. AJVR. 66(5), 755-761.

Hondalus, M.K., Mosser, D.M., 1994. Survival and replication of *Rhodococcus equi* in macrophages. Infect. Immun. 6, 24167–4175.

Jones, C.J.P., Jayson, M.I.V., 1984. Chloroquine: its effect on leucocyte auto- and heterophagocytosis. Ann. Rheum. Dis. 43, 205-212.

Jordan, M.C., Harrington, J.R., Cohen, N.D., Tsolis. R.M., Dangott, L.J., Weinberg, E.D., Martens, R.J., 2003. Effects of iron modulation on growth and viability of *Rhodococcus equi* and expression of virulence-associated protein A. Am. Journal. Vet. Res. 64(11), 1337-1346.

Krogstad, D.J., Schlesinger, P.H., 1987. The basis of antimalarial action: non-weak base effects of chloroquine on vesicle pH. Am. J. Trop. Med. Hyg. 36, 213-220.

Kwakye-Berko, F., Meshnick, S.R. 1989. Binding of chloroquine to DNA. Mol. Biochem. Parasitol. 35(1), 51-5.

Lane, T.E., Wu-Hsieh, B.A., Howard, D.H., 1991. Iron limitation and the gamma interferon-mediated antihistoplasma state of murine macrophages. Infect. Immun. 59, 2274–2278.

Lestas, A.N., 1976. The effect of pH upon human transferrin: selective labelling of the two iron-binding sites. Br. J. Haematol. 32:341-350.

Levitz, S.M., Harrison, T.S., Tabuni, A., Liu, X., 1997. Chloroquine induces human mononuclear phagocytes to inhibit and kill *Cryptococcus neoformans* by a mechanism independent of iron deprivation. ASCI. 100(6), 1640–1646.

Lin, L., Pantapalangkoor, P., Tan, B., Bruhn, K.W., Ho, T., Nielsen, T., Skaar, E.P., Zhang, Y., Bai, R., Wang, A., Doherty, T.M., Spellberg, B., 2014. Transferrin iron starvation therapy for lethal bacterial and fungal infections. J. Infect. Dis. 210(2), 254-264.

Matsusawa, Y., Hostetler, K.Y., 1980. Inhibition of lysosomal phospholipase A and phospholipase C by chloroquine and 4,4‘-Bis(diethylaminoethoxy) alpha, beta-diethyldiphenylethan. The J. Biol. Chem. 255, 5190-5194.

Miranda-CasoLuengo, R., Coulson, G.B., Miranda-CasoLuengo, A., Vázquez-Boland, J.A., Hondalus, M.K., Meijer, W.G., 2012. The hydroxamate siderophore rhequichelin is required for virulence of the pathogenic actinomycete *Rhodococcus equi*. Infect. Immun. 80(12), 4106-4114.

Newman, S.L., Gootee, L., Brunner, G., Deepe, G.S. Jr., 1994. Chloroquine induces human macrophage killing of *Histoplasma capsulatum* by limiting the availability of intracellular iron and is therapeutic in a murine model of histoplasmosis. J. Clin. Invest. 93, 1422–1429.

Núñez, M.T., Gaete, V., Watkins, J.A., Glass, J., 1990. Mobilization of iron from endocytic vesicles-the effects of acidification and reduction. J. Biol. Chem. 265, 6688–6692.

Octave, J.N., Scheneider, Y.J., Hoffman, P., Troluet, A., Crichton, R.R., 1979. Transferrin protein and iron uptake by culture rat fibroblasts. FEBS Lett. 108, 127–130.

Prescott, J.F., 1991. *Rhodococcus equi*: an animal and human pathogen. Clin. Microbiol. Rev. 29, 696–2700.

Prescott, J.F., Meijer, W.G., Vázquez-Boland, J.A., 2010. *Rhodococcus equi*. In: Gyles, C.L., Prescott, J.F., Songer, J.G., Thoen, C.O. (Eds), Pathogenesis of bacterial infections in animals, 4th ed, Wiley-Backwell, Ames, IA, pp. 149-166.

Princiotto, J.V., Zapolski, E.J., 1975. Difference between the two iron-binding sites of transferrin Nature. 255, 87-88.

Rahman, M.T., Herron, L.L., Kapur, V., Meijer, W.G., Byrne, B.A., Ren, J., Nicholson, V.M., Prescott, J.F., 2003. Partial genome sequencing of *Rhodococcus equi* ATCC 33701. Vet. Microbiol. 94, 143-158.

Ratledge, C., Dover, L.G., 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54, 881-941.

Ren, J., Prescott, J.F., 2003. Analysis of virulence plasmid gene expression of intra-macrophage and in vitro grown *Rhodococcus equi* ATCC 33701. Vet. Microbiol. 94, 167-182.

Schaible, U.E., Sturgill-Koszycki, S., Schlesinger, P.H., Russell, D.H., 1998. Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. J. Immunol. 160, 1290–1296.

Sibille, J., Kondo, H., Aisen, P., 1989. Uptake of ferritin and iron bound to ferritin by rat hepatocytes: modulation by apotransferrin, iron chelators and chloroquine. Biochem, Biophys, Acta. 1010, 204–208.

Sung, S.S.J., Nelson, R.S., Silverstein, S.C., 1983. Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. J. Cell. Biol. 96(1), 160-166.

Swaiman, K.F., Machen, V.L., 1986. Chloroquine reduces neuronal and glial iron uptake. J. Neurochem. 46, 652–654.

Tietze, C., Schlesinger, P., Stahl, P., 1980. Chloroquine and ammonium ion inhibit receptor-mediated endocytosis of mannose-glycoconjugates by macrophages: apparent inhibition of receptor recycling. Biochem. Biophys. Res. Commun. 93(1), 1-8.

Via, L.E., Fratti, R.A., McFalone, M., Pagan-Ramos, E., Deretic, D., Deretic, V., 1998. Effects of cytokines on mycobacterial phagosome maturation. J. Cell. Sci. 111, 897–905.

von Bargen, K., Wohlmann, J., Taylor, G.A., Utermöhlen, O., Haas, A., 2011. Nitric oxide-mediated intracellular growth restriction of pathogenic *Rhodococcus equi* can be prevented by iron. Infect. Immun. 79(5), 2098-111.

Weinberg, E.D., 1975. Nutritional immunity. Host's attempt to withold iron from microbial invaders. JAMA. 231(1), 39-41.

Yoon, Y.H., Cho, K.S., Hwang, J.J., Lee, S.J., Choi, J.A., Koh, J.Y., 2010. Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells. Invest. Ophthalmol. Vis. Sci. 51(11), 6030-6037.

Table 1. Oligonucleotides used in this study.

Gene	Nucleotide sequence	Purpose	Source of reference
gyrB-F	CGCTCACCTCGGTGGT	Ref. for normalization	This study
gyrB-R	TTGTCCTTGACGAGCTTCTTGT	Ref. for normalization	This study
16SrRNA-F	Pa04230899_s1_F*	Ref. for normalization	TaqMan Cat. # 4351372
16SrRNA-R	Pa04230899_s1_R*	Ref. for normalization	TaqMan Cat. # 4351372
rhbC-F	GGACGCCGAGGCTCTC	qPCR of *rhbC*	This study
rhbC-R	GCCAGCTCCCGGTAGGT	qPCR of *rhbC*	This study
vapA-F	AACGGTCGAGCAAGCGATAC	qPCR of *vapA*	Harrington et al., 2005
vapA-R	GGCCCGAATACGTGAAACCT	qPCR of *vapA*	Harrington et al., 2005

* Proprietary information for commercially-available product

Table 2. Effect of various CQ concentrations and pre-incubation time on the phagocytic capacity for *R. equi* of J774A.1 murine macrophages.

µM CQ	**Mean CFU's (95% Confidence Interval) Time (Hours)**			
	0.5	**1**	**2**	**4**
0	129,778[a] (120,531 – 139,734)	132,211[a] (122,166 – 143,082)	136,560[a] (126,184 – 147,788)	128,870[a] (119,079 -139,467)
10	124,853[a] (115,367 – 135,120)	127,194[a] (117,530 – 137,653)	131,378[a] (121,396 – 142,181)	123,980[a] (114,560 – 134,175)
20	125,127[a] (115,620 – 135,415)	127,473[a] (117,788 – 137,955)	131,666[a] (121,662 – 142,492)	124,252[a] (114,811 – 134,468)
50	95,830[b] (88,549 – 103,709)	100,838[b] (90,209 – 105,654)	97,627[b] (93,176 –109,129)	95,160[b] (87,929 – 102,984)

Note: The results are the CFU/per monolayer at each time; the mean and 95% CI values are linear mixed-effects model-derived estimates, and represent back-transformed results. Values within columns with different superscripted letters differ significantly ($P < 0.05$).

Table 3. Amount of superoxide anion (nmol/10^6 cells) in the supernatant by time, CQ concentration, and exposure to *R. equi*.

Time (*h*)	Cytochrome C Activity (95% Confidence Interval)					
	Chloroquine Concentration (μM)					
	0 No RE	***0 + RE***	***10 No RE***	***10 +RE***	***20 No RE***	***20 + RE***
0	6.15^{a} (5.83 – 6.48)	NA	5.79^{b} (5.44 - 6.14)	NA	6.11^{ab} (5.76 – 6.46)	NA
12	6.11^{ab} (5.79 – 6.44)	6.28^{a} (5.95 – 6.60)	NA	6.88^{c} (6.40 -7.36)	NA	6.28^{ab} (5.80 – 6.76)
24	6.04^{ab} (5.71 – 6.37)	6.20^{a} (5.88 – 6.53)	NA	6.23^{ab} (5.75–6.71)	NA	6.36^{ab} (5.88 – 6.84)

Note: Values with different superscripted letters differ significantly ($P < 0.05$); the mean and 95% CI values are model-derived estimates. RE – *R. equi*, NA - not applicable

Table 4. Lysozyme activity by time, CQ concentration, and *R. equi* presence.

Time (Hrs)	**Lysozyme Activity (95% Confidence Interval)**					
	Chloroquine Concentration (µM)					
	0 No RE	***0 + RE***	***10 No RE***	***10 +RE***	***20 No RE***	***20 + RE***
0	249.5[a] (220.5 – 277.6)	NA	214.8[b] (188.0 -241.5)	NA	204.8[b] (177.8 - 231.7)	NA
12	107.0[c] (71.4 – 142.6)	100.6[c] (64.9 -136.2)	NA	66.3[d] (30.7 – 101.9)	NA	56.3[d] (20.7 – 91.3)
24	349.4[e] (318.6 – 380.3)	343.0[e] (307.5 – 378.6)	NA	308.8[f] (273.2 – 344.3)	NA	298.8[f] (263.2 – 34.4)

Note: Values with different superscripted letters differ significantly ($P < 0.05$); the mean and 95% CI values are model-derived estimates. RE – *R. equi*, NA - not applicable

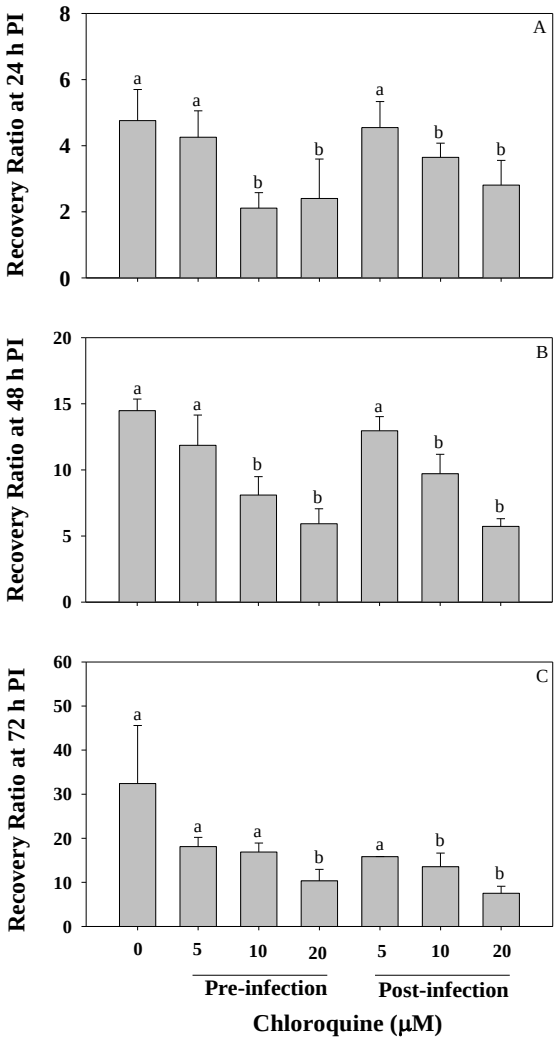

Fig. 1 Chloroquine decreased the number of viable *R. equi* in J774A.1 macrophages. The macrophages were treated for 24 h pre-infection or treated post-infection with 5, 10, and 20 μM CQ. A) *R. equi* recovery ratio (RR) at 24 h post-infection. B) *R. equi* RR at 48 h post-infection. C) *R. equi* RR at 72 h post-infection. The horizontal axis represents the CFU of RR, where RR was defined by the equation RR= (mean CFU recovered at a given time)/(mean CFU recovered at time 0 h). Groups with letters different than "a" differ significantly ($P < 0.05$) from the control group (0 μM CQ). PI = post-infection

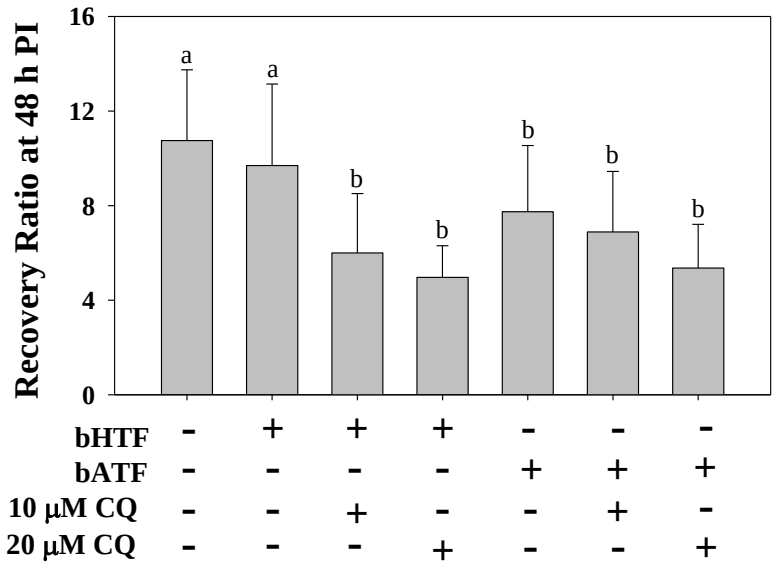

Fig. 2 bHTF does not reverse the inhibition of *R. equi* intracellular survival due to CQ treatment. The number of *R. equi* CFU was verified at 48 h post-incubation. Groups with letters different than "a" differ significantly ($P < 0.05$) from the control group (0 μM CQ). See Fig. 2 for key.

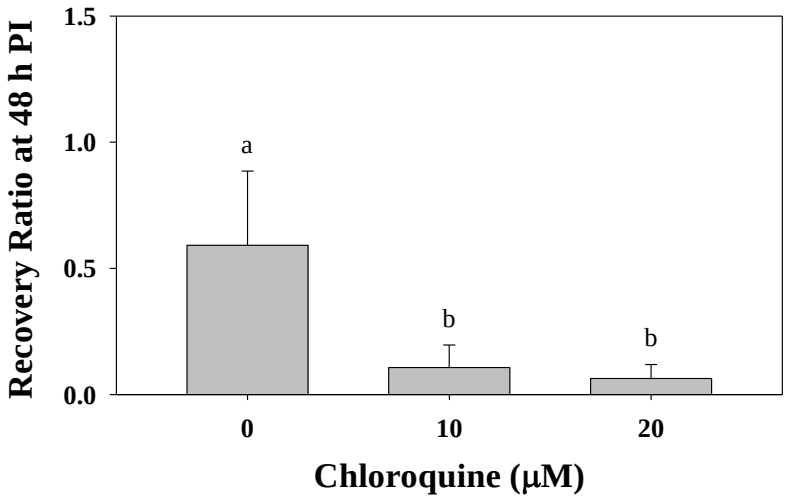

Fig. 3 Chloroquine significantly decreased the number of viable *R. equi* in foal AMs. The AMs were pretreated for 24 h prior to infection with *R. equi*. The recovery ratio (48 h CFU/ 0 h CFU) were plotted by CQ treatment group (i.e., 10 or 20 μM CQ). For each foal, the mean of duplicate wells was used for analysis. Results were expressed as the fold-reduction in the number of *R. equi* CFU/well relative to the uninfected control monolayers of each foal. Groups with letters different than "a" differ significantly ($P < 0.05$) from the control group (0 μM CQ).

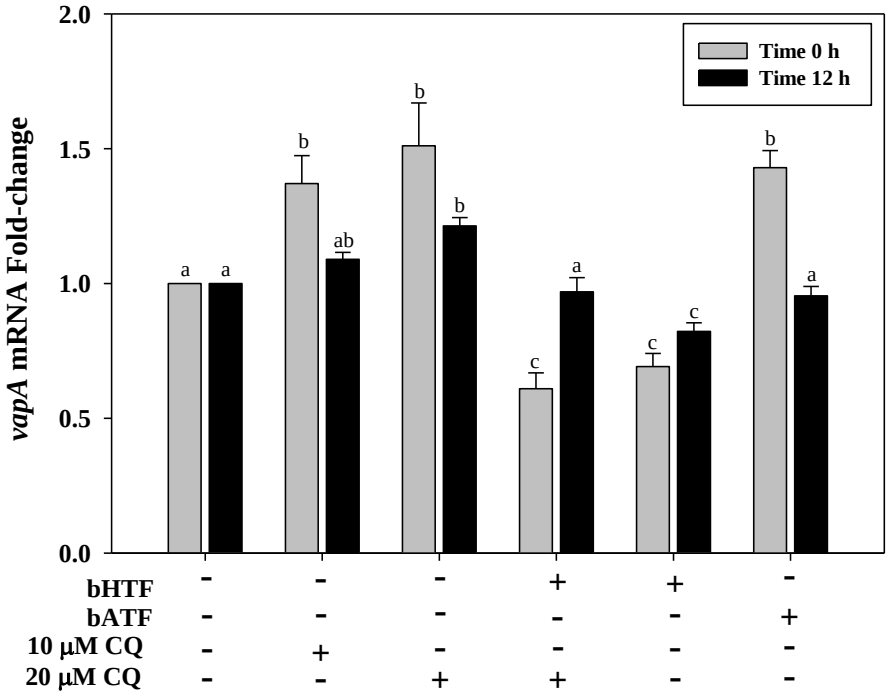

Fig. 4 Chloroquine treatment induced relative *vapA* transcript level at 0 h and 12 h post-infection. Fold-change of *vapA* transcript level at 0 h (1 h after addition of medium supplemented with gentamicin) and 12 h post-infection. J774A.1 macrophages were pretreated for 24 h before infection with *R. equi* (MOI 20). All mRNA changes are given relative to control cells (data from 3 independent experiments performed in duplicate). Groups with letters different than "a" differ significantly ($P < 0.05$) from the control group (0 µM CQ).

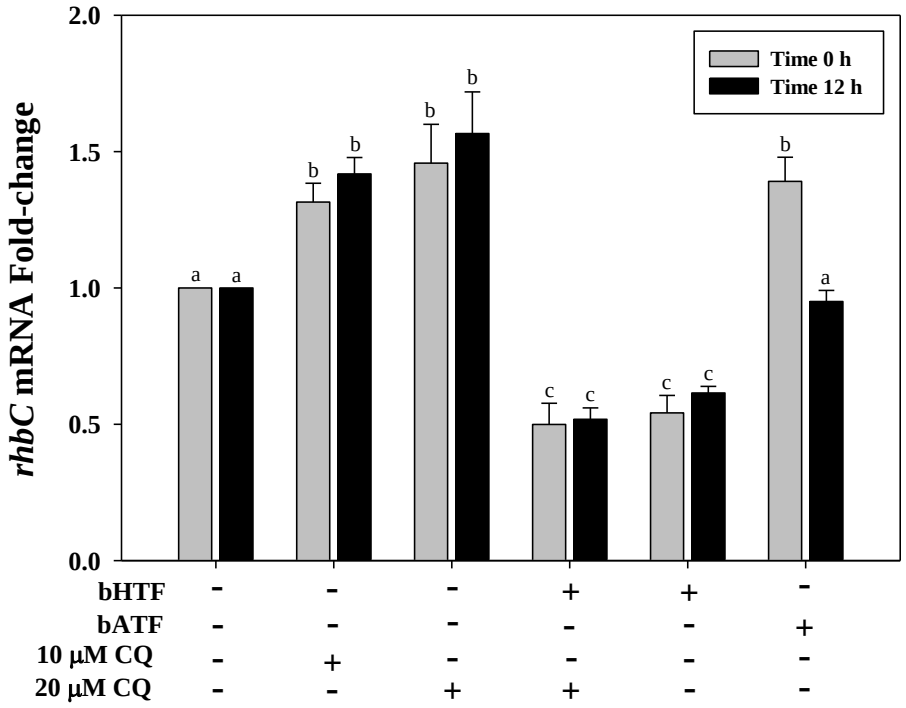

Fig. 5 Chloroquine treatment induced *rhbC* expression at 0 h post-infection (1 h after addition of medium supplemented with gentamicin) and 12 h post-infection. Results of fold-changes of *rhbC* expression at 0 h and 12 h post-infection are portrayed. J774A.1 murine macrophages were pretreated for 24 h prior to infection with *R. equi* (MOI 20). All mRNA fold-changes are relative to control cells (data from 3 independent experiments performed in duplicate). Groups with letters different than "a" differ significantly ($P < 0.05$) from the control group (0 µM CQ).

4. MANUSCRITO 3

Research paper

Genetic susceptibility to *Rhodococcus equi* in Brazilian Sport horses and Brazilian Thoroughbreds

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(Artigo a ser submetido para publicação – Plos One)

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Genetic susceptibility to *Rhodococcus equi* in Brazilian Sport horses and Brazilian Thoroughbreds

Abstract

Rhodococcus equi is a major cause of foal pneumonia. Among the factors associated with *R. equi* occurrence, indices of genetic susceptibility have been reported, including a recent study from our laboratory. To investigate possible genetic contributions to *R. equi* pneumonia, we tested 60 DNA samples for genetic loci associated with pneumonia from 2 horse breeds (viz., Brazilian Sport Horse and Brazilian Thoroughbred) not previously studied using the Illumina EquineSNP70 BeadChip Array. The SNP-based genome-wide association study (GWAS) identified regions on chromosomes 10, 11, 16, and 28 that had moderate evidence of association with *R. equi* pneumonia. These regions contain important genes, such as GNB2L1 (guanine nucleotide-binding protein subunit beta-2-like 1), FUCA2 (α-L-fucosidase 2), ABR (active BCR-related), and TIMP3 (endogenous metalloprotease inhibitor) which are involved in immune responses to intracellular pathogens including neutrophil degranulation, modulation the intensity of early inflammatory responses, and regulation of phagocytosis. Results of this study using breeds not previously evaluated by GWAS substantiate the crucial role of innate immune responses in the pathogenesis of *R. equi* pneumonia. Findings of this study also affirm the polygenic basis of susceptibility to *R. equi* pneumonia. It is plausible that a variety of variations in innate immune genes result in permutations and combinations of functional innate responses that predispose foals to *R. equi* pneumonia.

1. Introduction

Rhodococcus equi is a gram-positive, facultative intracellular coccobacillus that predominantly affects young foals and that causes chronic, abscessing bronchopneumonia or extrapulmonary disorders that can be fatal (Prescott, 1991; Prescott et al., 2010). Currently, no approved vaccine exists to protect against *R. equi* pneumonia, and other preventative interventions such as transfusion of hyperimmune plasma are expensive, labor-intensive, and incompletely effective (Giguère et al., 2011; Venner et al., 2009). *Rhodococcus equi* is a soil saprophyte organism that is known to occur ubiquitously in the environment, especially at farms where equine rhodococcosis occurs sporadically or endemically. Even though exposure is widespread at endemic farms, it remains unclear why some foals are healthy, some foals show only subclinical signs, and some foals develop severe clinical signs of pneumonia. Anecdotal evidence describes some mares having multiple affected foals while other mares in the same environment never have an affected foal. These observations along with several candidate gene associations suggest that a genetic predisposition to *R. equi* pneumonia might exist (Halbert et al., 2006; Horin et al., 2004; McQueen et al., 2014; Mousel et al., 2003).

Pneumonia caused by *R. equi* is a complex trait that appears to have a polygenic basis (McQueen et al., 2015). The associations from candidate gene studies, which support a polygenic model, have been weak and potentially biased. Recently, we conducted a genome-wide association study (GWAS) among Quarter Horse foals from the United States classified as having clinical *R. equi* pneumonia or subclinical *R. equi* pneumonia, or being healthy. In these foals, a gene on chromosome 26 was associated with clinical pneumonia caused by *R. equi* (McQueen et al., 2014). The transient receptor potential cation channel (*TRMP2*) gene was implicated as a plausible candidate gene because its documented association with chemotaxis and neutrophilic activity (Yamamoto et al., 2008). Neutrophils are known to play an important role in *R. equi* clearance by influencing lung burdens of *R. equi* and lung lesions:

neutrophil-induced deficiency resulted in more severe disease and significantly increased tissue concentrations of *R. equi* in mice experimentally infected with *R. equi* (Martens et al., 2005). These GWAS results further strengthened evidence of a genetic contribution to predisposition to *R. equi* foal pneumonia.

In many cases, SNPs are merely indicators of genetic location of a causal variant and are very rarely the site of the actual causal mutation(s). Thus, it remains unclear if more genes or other genetic elements (e.g., promoters and silencers) in these previously studied loci or genes explain the observed associations of genotype with disease. Identification of genotype associations with *R. equi* pneumonia outside of previously investigated breeds would strengthen our understanding of the genetic basis of susceptibility to *R. equi*. Therefore, the objective of this study was to better understand the genetic basis of *R. equi* susceptibility in the Brazilian Sport Horse (BSH) and Brazilian Thoroughbred (BTB) breeds.

The objective of this study was to test the hypothesis that there would be regions of the genome associated with either resistance or susceptibility to *R. equi* pneumonia in these genetically diverse breeds of horses which would shed further light on critical pathways and biological processes in the pathogenesis of equine rhodococcosis. To accomplish this objective, we investigated the association of *R. equi* pneumonia in BSH and BTB breeds with previously identified genes or genetic variants, as well as identifying new associations using an unbiased GWAS approach, using the equine SNP array.

2. Material and Methods

2.1 Ethical statement

All procedures for this study were assessed and approved by the Ethics Committee of Animal Use (Protocol 133/2014), Federal University of Santa Maria, Brazil. Documents

attesting the consent to participation in the study by providing samples as well as access to animals and medical history were provided by animal owners.

2.2 Study population

The study population was comprised of 60 horses comprised of 31 BSH and 29 BTB from Rio Grande do Sul, Brazil. The horses selected for this study were from farms that had history of recurrent *R. equi* pneumonia. The following criteria of clinical signs were considered to be suggestive of *R. equi* induced pneumonia: fever, lethargy, cough, nasal discharge, polysynovitis, tachypnea, increased respiratory effort, respiratory distress, and pulmonary crackles or wheezes detected by thoracic auscultation. Foals that had at least 3 of the aforementioned clinical signs and had evidence of focal pulmonary consolidation or pulmonary abscessation (evidenced by ultrasonography examination) were considered to have *R. equi* pneumonia. Foals that did not develop clinical signs of pneumonia were classified as unaffected (control) foals; no control foals had thoracic ultrasonographic evidence of pulmonary consolidation or abscessation when examined. Ultrasonographic screening was not performed at the participating farms (n = 5), such that subclinical cases of pneumonia were not identified for this study. The number of foals included in this study was determined by available funding and the costs of the SNP array. Results from of our previous study indicated that we could anticipate adequate power for to detect odds ratios (ORs) > 2 for SNPs with minor allele frequencies > 5% (McQueen et al., 2014).

2.3 DNA sampling

Venous blood was collected by jugular venipuncture in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing EDTA. Genomic DNA was isolated from

whole blood leukocytes using a DNeasy® Blood & Tissue Kit (Qiagen®, Cat No. 69506) according to the manufacturer's protocol and stored at -20° C until use. All DNA samples used in the study had spectrophotometric ratios of A260/A280 between 1.7 and 1.9.

2.4 SNP genotyping

The SNP genotyping was performed commercially by GeneSeek (Neogen, Lincoln, NE) using the EquineSNP70 BeadChip Array (Illumina, San Diego, CA). The resulting SNP genotypes were analyzed using the PLINK analysis package (Purcell et al., 2007). Genotype ped files were loaded into PLINK and foals were assigned a phenotypic status of either affected or unaffected (i.e., case of pneumonia or healthy control) based on their prior diagnosis and breed (**Figure 1**.).

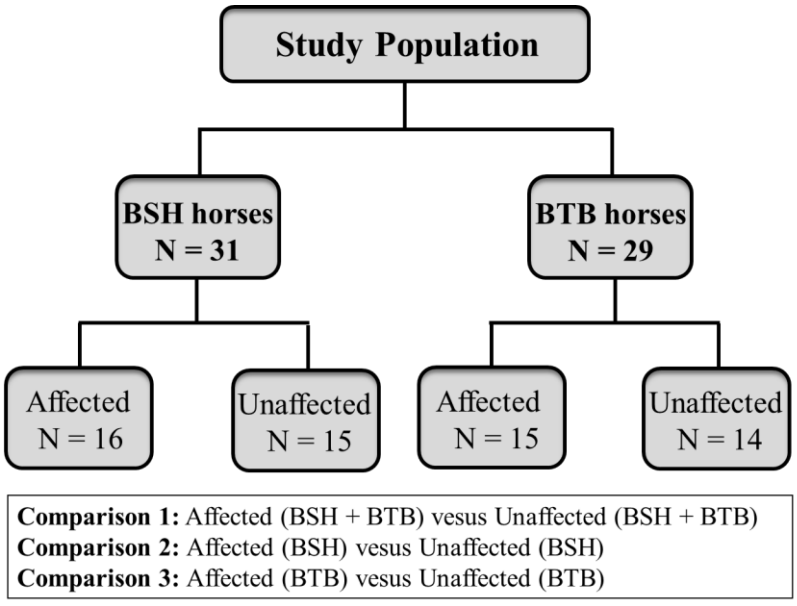

Figure 1. Schematic of the population of by breed (Brazilian sport horses [BSH] and Brazilian Thoroughbreds [BTB]) and disease phenotype (affected with pneumonia or unaffected). Association of SNPs with disease phenotype was determined for both breeds combined and each individual breed independently.

Genotypes were determined for each animal and then filtered (i.e., samples excluded) on the basis of missingness per individual (> 10%), missingness per SNP (> 10%), minor allele frequency (< 5%), and absence of Hardy-Weinberg equilibrium (P < 0.001), as described previously (Raudsepp et al., 2012). A standard chi-squared association test (Max (T) permutations [N = 10,000]) based on a binary outcome of disease status using a case-control design was performed using PLINK (Raudsepp et al., 2012). A P value < 1×10^{-4} was considered evidence of association (Wellcome Trust Case Control, 2007).

Population stratification was determined using plots of the observed versus the expected -$\log_{10}$ P values of chi-squared association tests and by determining the genomic inflation factor using the R package GenABEL (Aulchenko et al., 2007). Using PLINK, mixed-effects logistic regression with farm modeled as a random effect was used for the association test in comparisons showing evidence of population stratification; SNPs with any genotype represented fewer than 10 times were removed from analysis to permit model convergence (Aulchenko et al., 2007; Price et al., 2010). All SNP array data have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through GEO Series accession.

2.5 SNPs investigation and genes analysis

To classify the location of SNPs in coding sequences of genes, non-coding regions of genes, or in the intergenic regions we used the UCSC Genome Browser database (available at https://genome.ucsc.edu). To investigate the molecular and biological functions of genes for which SNPs were positively associated with disease, we used the PANTHER classification system (www.pantherdb.org). In addition to the database analyses, functional information was also obtained from a literature search.

3 Results

3.1 Plink data

The number of SNPs excluded on the basis of missingness per individual, missingness per SNP, and minor allele frequency were 0, 334, and 10,695, respectively (65,157 total SNPs). For comparisons 1, 2, and 3 (**Figure 1**), the number of SNPs excluded on the basis of Hardy-Weinberg equilibrium were 200, 58, and 29, respectively. After filtering, the total genotyping rate of the foals was estimated at 99.5%.

In the present GWAS study we identified SNPs that segregated according to the phenotypes evaluated. These results allowed us to identify regions of interest to further investigate if genes, promoters, or other elements were associated with affected and unaffected foals. Using the option for association studies, the distributions of SNPs were compared between the comparisons groups (**Figure 1**). The results from this study are represented graphically in a Manhattan plot (**Figure 2**) showing the P values for the evaluated SNPs distributed by the equine chromosomes.

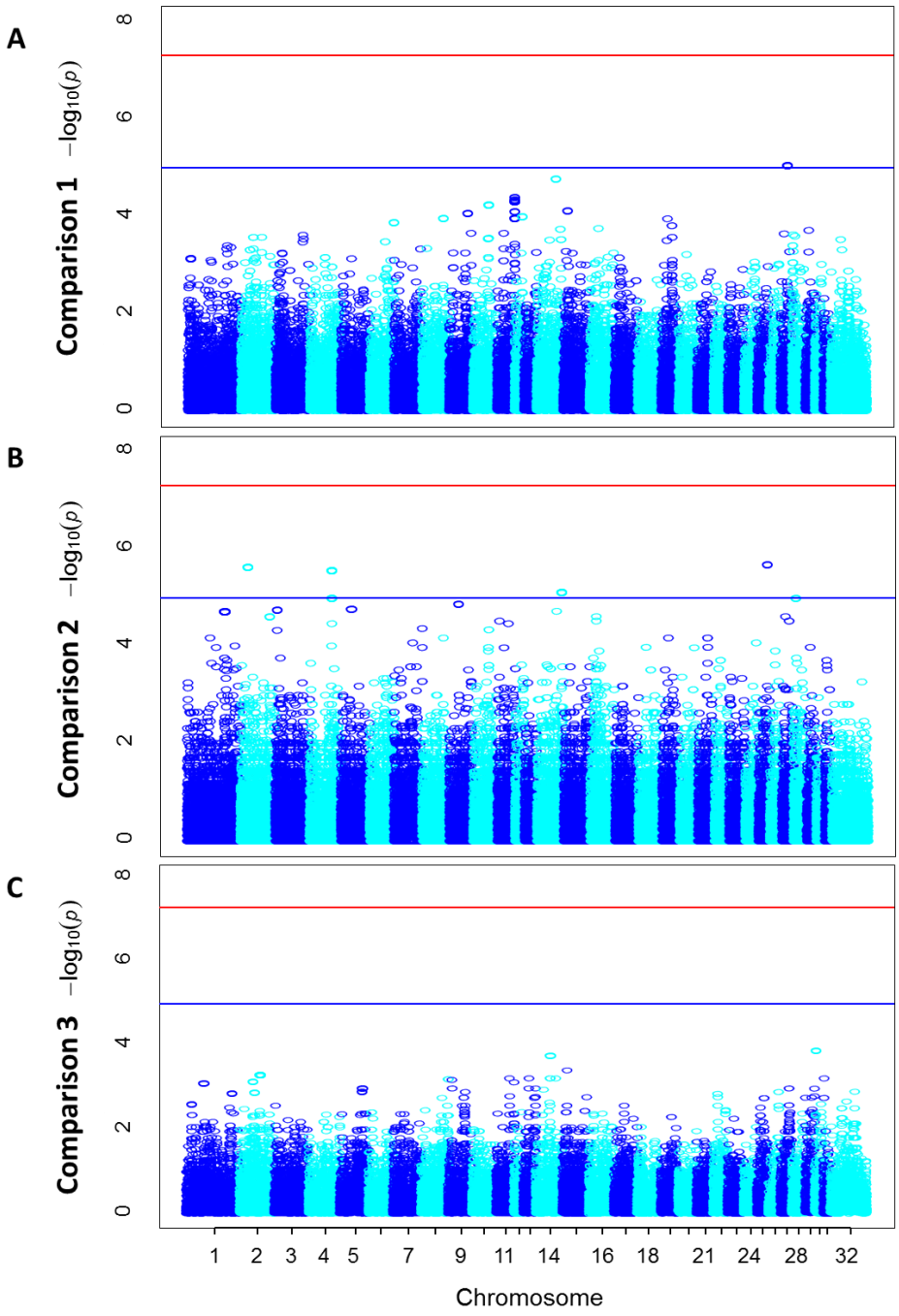

Figure 2. Manhattan plots of standard chi-squared significance values for the 3 genome-wide comparisons studies. (A) Comparison 1, (B) Comparison 2, and (C) Comparison 3. Genomic positions are indicated by chromosomes with different number.

There were 127 SNPs moderately associated with clinical pneumonia based on P value < 10^{-4} (Table 1). Results for comparison 1 (affected BSH + BTB [N=31] vs. unaffected BSH +

BTB [N=29]; λ=1.23) identified 46 SNPs associated with clinical pneumonia, comparison 2 (affected BSH [N=16] vs. unaffected BSH [N=15] λ=1.54) identified 69 SNPs, and comparison 3 (affected BT [N=15] vs. unaffected BT [N=14] λ=1.02) identified 12 SNPs.

The λ value of comparison 2 (1.54) was evidence of confounding population structure and inspection of sampling locations suggested it might have been farm-related. Thus, mixed-effects modeling with farm considered as a random-effect term was used to adjust the resulting inflated association statistics.

Table 1. Moderately associated SNPs for each genome-wide comparison study.

Comparison 1				
SNP ID	**Chromosomal location**	**P value**	**Odds Ratio**	**Localization**
BIEC2_289433	chr15:14742827-14742829	7.84E-05	5.64	Intergenic
BIEC2_1101485	chr9:65403911-65403913	8.60E-05	6.609	Intronic
BIEC2_1062453	chr8:72275100-72275102	1.10E-04	4.804	Intronic
BIEC2_453178	chr19:19939617-19939619	1.12E-04	6	Intronic
BIEC2_724840	chr28:3555388-3555390	2.38E-04	4.623	Intergenic
TBIEC2_764670	chr28:9516259-9516261	2.55E-04	4.51	Intronic
BIEC2_487431	chr2:71092983-71092985	2.69E-04	4.333	Intergenic
BIEC2_437253	chr19:36213078-36213080	2.69E-04	4.333	Intronic
BIEC2-804055	chr3:95582159-95582161	2.90E-04	5.143	Intronic
BIEC2_122878	chr10:51177587-51177589	2.90E-04	5.143	Intergenic
BIEC2_122890	chr10:51185836-51185838	2.90E-04	5.143	Intergenic
BIEC2_1119755	chr32:33940510-33940512	3.00E-04	7.294	Intergenic
BIEC2_430702	chr19:17982121-17982123	3.14E-04	10.95	Intronic
UKUL1471	chr6:52969964-52969966	3.32E-04	18.33	Exonic
BIEC2_164877	chr11:60237956-60237958	3.74E-04	5.645	Intergenic
TBIEC2_760109	chr28:1505466-1505468	3.99E-04	4.6	Intergenic
BIEC2_723787	chr28:1637826-1637828	3.99E-04	4.6	Intergenic
BIEC2_723788	chr28:1638101-1638103	3.99E-04	4.6	Intergenic
BIEC2_162925	chr11:44533142-44533144	4.79E-04	5.133	Intergenic
BIEC2_1121351	chr32:38507117-38507119	4.87E-04	7	Intergenic
BIEC2_490988	chr2:30194321-30194323	4.91E-04	4.278	Intergenic
BIEC2_491001	chr2:30284884-30284886	4.91E-04	4.278	Intronic
BIEC2_491088	chr2:30386622-30386624	4.91E-04	4.278	Intronic
BIEC2_724869	chr28:3693341-3693343	5.25E-04	4.171	Intergenic
BIEC2_714993	chr27:30207302-30207304	5.33E-04	4.63	Intergenic
BIEC2_1074947	chr9:12697924-12697926	5.64E-04	5.873	Intronic
BIEC2_773954	chr3:20931335-20931337	5.66E-04	3.985	Intronic

BIEC2_1095585	chr9:55241507-55241509	5.66E-04	3.985	Intergenic
BIEC2_364874	chr16:82278535-82278537	5.66E-04	3.985	Intronic
BIEC2_774057	chr3:21799558-21799560	5.88E-04	4.759	Intronic
BIEC2_865840	chr4:51725166-51725168	6.96E-04	4.476	Intronic
BIEC2_123217	chr10:51956428-51956430	6.96E-04	4.476	Intergenic
BIEC2_865805	chr4:51639035-51639037	7.08E-04	4.222	Intronic
BIEC2_476359	chr2:45082430-45082432	7.52E-04	5.208	Intergenic
BIEC2-973348	chr7:258729-258731	7.99E-04	9.722	Intergenic
BIEC2_34426	chr1:78983404-78983406	8.02E-04	6.314	Intronic
BIEC2_1016659	chr6:71485121-71485123	8.02E-04	6.314	Intergenic
BIEC2_648037	chr24:36626228-36626230	8.74E-04	4.56	Intergenic
BIEC2_331272	chr16:18885331-18885333	8.95E-04	4.091	Intronic
BIEC2_723814	chr28:1673640-1673642	8.95E-04	4.091	Intergenic
BIEC2_723816	chr28:1673753-1673755	8.95E-04	4.091	Intergenic
BIEC2-723972	chr28:1940779-1940781	8.95E-04	4.091	Intergenic
BIEC2_760572	chr28:2144446-2144448	8.95E-04	4.091	Intergenic
BIEC2_724153	chr28:2147441-2147443	8.95E-04	4.091	Intergenic
BIEC2-724179	chr28:2218059-2218061	8.95E-04	4.091	Intergenic
BIEC2_173306	chr11:60586459-60586461	9.06E-04	3.896	Intergenic

Comparison 2

SNP ID	Chromosomal location	P value	Odds Ratio	Localization
BIEC2_870143	chr4:75240694-75240696	2.74E-06	17.88	Intergenic
BIEC2_915357	chr4:75571083-75571085	2.74E-06	17.88	Intergenic
BIEC2_870228	chr4:75658975-75658977	2.74E-06	17.88	Intergenic
BIEC2_870139	chr4:75235754-75235756	1.03E-05	13.75	Intergenic
BIEC2_870142	chr4:75239582-75239584	1.03E-05	13.75	Intergenic
TBIEC2_764670	chr28:9516259-9516261	1.03E-05	13.75	Intronic
BIEC2_331986	chr16:20094492-20094494	2.48E-05	13	Intronic
BIEC2_870266	chr4:76181702-76181704	3.47E-05	11	Intergenic
BIEC2_150571	chr11:36970315-3697031	3.47E-05	11	Intergenic
BIEC2_34426	chr1:78983404-78983406	6.81E-05	11.23	Intronic
BIEC2_1062453	chr8:72275100-72275102	6.81E-05	11.23	Intronic
BIEC2_453178	chr19:19939617-19939619	6.81E-05	11.23	Intronic
BIEC2_561268	chr21:31935031-31935033	6.81E-05	11.23	Intronic
BIEC2_561271	chr21:31946542-31946544	6.81E-05	11.23	Intronic
BIEC2_1004778	chr7:63954523-63954525	8.57E-05	10	Intergenic
BIEC2_649102	chr24:37777266-37777268	8.57E-05	10	Intergenic
TBIEC2_45870	chr1:100029084-100029086	1.07E-04	9.036	Intronic
TBIEC2_1075632	chr7:97277417-97277419	1.07E-04	9.036	Intronic
BIEC2_122878	chr10:51177587-51177589	1.07E-04	9.036	Intergenic
BIEC2_122890	chr10:51185836-51185838	1.07E-04	9.036	Intergenic
BIEC2-36465	chr1:84090209-84090211	2.25E-04	8.636	Intronic
BIEC2_589859	chr21:31844614-31844616	2.25E-04	8.636	Intergenic
BIEC2_729314	chr28:11388606-11388608	2.25E-04	8.636	Intergenic
BIEC2_255738	chr14:36766816-36766818	2.65E-04	8	Intronic
BIEC2_273182	chr14:87408234-87408236	2.65E-04	8	Intergenic

BIEC2_294206	chr15:21347284-21347286	2.65E-04	8	Intergenic
BIEC2_340990	chr15:88430128-88430130	2.65E-04	8	Intronic
BIEC2_81429	chr1:170504303-170504305	2.87E-04	13.85	Intronic
BIEC2_331204	chr16:18727036-18727038	2.91E-04	7.667	Intronic
BIEC2_331272	chr16:18885331-18885333	2.91E-04	7.667	Intronic
BIEC2_123217	chr10:51956428-51956430	3.01E-04	7.562	Intergenic
BIEC2_1101485	chr9:65403911-65403913	3.09E-04	10.29	Intronic
BIEC2_833583	chr31:7043413-7043415	3.09E-04	10.29	Intronic
BIEC2_136031	chr11:5770432-5770434	4.34E-04	8.5	Intronic
BIEC2_558330	chr21:25545502-25545504	4.34E-04	8.5	Intergenic
BIEC2_953055	chr6:47395272-47395274	4.60E-04	12.25	Exonic
BIEC2_958816	chr6:58657543-58657545	4.60E-04	12.25	Intergenic
BIEC2-197231	chr12:28259099-28259101	4.60E-04	12.25	Intergenic
BIEC2_1148217	chr32:102513220-102513222	5.42E-04	15.43	Intergenic
BIEC2_265881	chr14:32246168-32246170	5.57E-04	7.5	Intergenic
BIEC2_347892	chr16:20576276-20576278	5.57E-04	7.5	Intronic
BIEC2_990348	chr6:15036048-15036050	5.67E-04	19.33	Intergenic
UKUL1471	chr6:52969964-52969966	5.67E-04	19.33	Exonic
BIEC2_292205	chr15:17649191-17649193	5.67E-04	19.33	Intergenic
BIEC2_430702	chr19:17982121-17982123	5.67E-04	19.33	Intronic
BIEC2_775036	chr28:30025912-30025914	5.67E-04	19.33	Intronic
BIEC2_458761	chr2:16894636-16894638	6.10E-04	7	Intergenic
BIEC2_869932	chr4:73650196-73650198	6.57E-04	6.937	Intronic
BIEC2_85106	chr1:176577995-176577997	6.64E-04	6.909	Intergenic
BIEC2_104653	chr10:11267857-11267859	6.64E-04	6.909	Intronic
BIEC2_330687	chr16:17542692-17542694	6.64E-04	6.909	Intergenic
BIEC2_700428	chr27:3172278-3172280	6.64E-04	6.909	Intronic
BIEC2_312432	chr15:59792427-59792429	7.23E-04	9	Intergenic
BIEC2_312439	chr15:59796220-59796222	7.23E-04	9	Intronic
BIEC2-348556	chr16:50228559-50228561	7.23E-04	9	Intergenic
BIEC2_772511	chr3:12157336-12157338	7.42E-04	6.571	Intergenic
BIEC2_97706	chr10:7657686-7657688	7.42E-04	6.571	Intronic
TBIEC2_162526	chr11:43937485-43937487	7.42E-04	6.571	Intronic
BIEC2_196172	chr12:26051157-26051159	7.42E-04	6.571	Intronic
BIEC2_740452	chr27:11727350-11727352	7.42E-04	6.571	Intergenic
BIEC2_763944	chr28:8406222-8406224	7.42E-04	6.571	Intronic
TBIEC2_765744	chr28:11002852-11002854	7.42E-04	6.571	Intergenic
BIEC2_364874	chr16:82278535-82278537	7.84E-04	6.417	Intronic
TBIEC2_561875	chr20:46272009-46272011	7.84E-04	6.417	Intergenic
BIEC2_740404	chr27:11666769-11666771	7.84E-04	6.417	Intergenic
BIEC2_737985	chr28:30982650-30982652	7.84E-04	6.417	Intronic
BIEC2_837967	chr31:15490965-15490967	7.84E-04	6.417	Intronic
BIEC2_837980	chr31:15495356-15495358	7.84E-04	6.417	Intronic
BIEC2_1012517	chr6:61687421-61687423	9.51E-04	9	Intergenic

Comparison 3

SNP ID	Chromosomal location	P value	Odds Ratio	Localization

BIEC2_814160	chr30:3391789-3391788	1.29E-04	17.33	Intronic
BIEC2_289433	chr15:14742828-14742827	3.73E-04	14.4	Intergenic
BIEC2_486338	chr2:69092143-69092142	4.97E-04	11.07	Intronic
BIEC2_487259	chr2:70769921-70769920	4.97E-04	11.07	Intronic
BIEC2_162925	chr11:44533143-44533142	5.80E-04	9.625	Intergenic
BIEC2_224026	chr13:18391284-18391283	5.80E-04	9.625	Intronic
CUHSNP00146844	chr14:50468965-50468964	5.80E-04	9.625	Exonic
BIEC2_1066485	chr8:92846498-92846497	6.09E-04	9	Intergenic
BIEC2_1066580	chr8:93347274-93347273	6.09E-04	9	Intergenic
BIEC2_1066587	chr8:93377057-93377056	6.09E-04	9	Intergenic
BIEC2_1066590	chr8:93392635-93392634	6.09E-04	9	Intergenic
BIEC2_1134021	chr9:11625980-11625979	6.32E-04	20.83	Intergenic

3.2. Analysis of SNPs moderately associated with *R. equi* clinical pneumonia

We initially performed an individual analysis of each SNP from **Table 1** that were classified into exonic, intronic, or intergenic regions. The majority of the SNPs is located into intergenic regions (n = 69), which were explored to find genes up-stream and down-stream (±50 Kb) each SNP. This search resulted in 20 SNPs classified in to intergenic regions that were located nearby genes. The remainder of the SNPs were distributed between intronic (n= 54) and exonic (n = 4) regions, totalizing 127 SNPs. This analysis leads us to 78 SNPs associated in some level to equine genes. After investigating the SNPs (n = 78) distribution we performed the following analysis by chromosomal regions positively associated to disease.

Chromosomal regions were analyzed and the genes found were evaluated regarding their molecular and biological functions. We analyzed specific regions into chromosome 2, 4, 10, 11, 15, 16, 21, 28, and 31 using the PANTHER classification database (**Table 2**). Based on the knowledge of the genes biological function we selected their known mechanism potentially associated to *R. equi* infection (**Table 2**). Genes into regions of chromosomes 11, verified in all comparisons (combined breeds, BSH only, and BTB only), 10, verified in comparisons 1 and 2 (combined breeds and BSH only), 16 and 28 verified in comparison 2 (only BHS) showed a strong potential to be associated to *R. equi* pneumonia based on their known mechanisms described in *Equus caballus*.

Table 2. List of genes located into chromosomal regions moderately associated to *R. equi* susceptibility.

Chr	Region	Comparison	Gene symbol[a]	Biological function[b]	Known mechanism potentially associated to *R. equi*[b]
2	30194321-30386622	1	RUNX3/1	biological regulation, cellular process, developmental process, immune system process, metabolic process, multicellular organismal process	-
4	51639085-51725168	1	TMEM196, LOC, TWISTNB	cellular process, metabolic process	-
4	75235754-76181702	2	LSM8, ANKRD7	cellular process, metabolic process	-
10	51177587-51956428	1; 2	MCHR2, SIM1/2, ASCC3, GNB2L1, FCRG2B, FUCA2	biological regulation, cellular process, developmental process, location, metabolic process	positive regulation of apoptotic process (GNB2L1), response to bacterium and regulation of entry of bacterium into host cell (FUCA2)
11	60237956-60586461	1	PEMT, RASD, MED9	metabolic process	-
11	44533142-43937485	1; 2; 3	GOSR1, ABR, TIMM22, TUSC5, BLMH, CPD	biological regulation, cellular process, location, metabolic process, response to stimulus	negative regulation of cellular extravasation, neutrophil degranulation, inflammatory response, and cell migration, response to lipopolysaccharide, positive regulation of phagocytosis (ABR), and response to drug (BLMH)
15	59792427-59796220	2	RMDN2	-	-
16	18727036-18885331	2	FOXP1, MIR1	biological regulation, cellular process, metabolic process	T follicular helper cell differentiation, regulation of lung goblet cell differentiation, lung secretory cell differentiation, immunoglobulin V(D)J recombination, lung development, pre-B cell differentiation (FOXP1)

16	20096492-20576276	2	MITF	metabolic process	cell fate commitment, negative regulation of apoptotic process, mast cell migration, regulation of apoptotic process
21	31844614-31946542	2	ZFR	apoptotic process, biological regulation, cellular process, developmental process, immune system process, metabolic process, multicellular organismal process, response to stimulus	-
28	30025912-30982650	2	BTBDD, ABTB2, PWP1, PRDM, FBX07, SYN1, TIMP3	biological regulation, cellular process, location, metabolic process, multicellular organismal process, response to stimulus	response to cytokine (TIMP3)
31	15490965-15495356	2	CCDC170, AKAP12	-	-

[a] Data from UCSC Genome Browser (horse).
[b] Data from PANTHER classification database.

We also analyzed the genes listed in **Table 2** by their molecular function and biological process using the PANTHER classification database (**Figures 3A** and **3B**). The principal categories of molecular function included binding, catalytic activities, and nucleic acid binding transcription factor activity, suggesting that these genes might mediate *R. equi*-host cell interactions (**Figure 3A**). Furthermore, there was a predominance of genes regulating metabolic and cellular processes (**Figure 3B**).

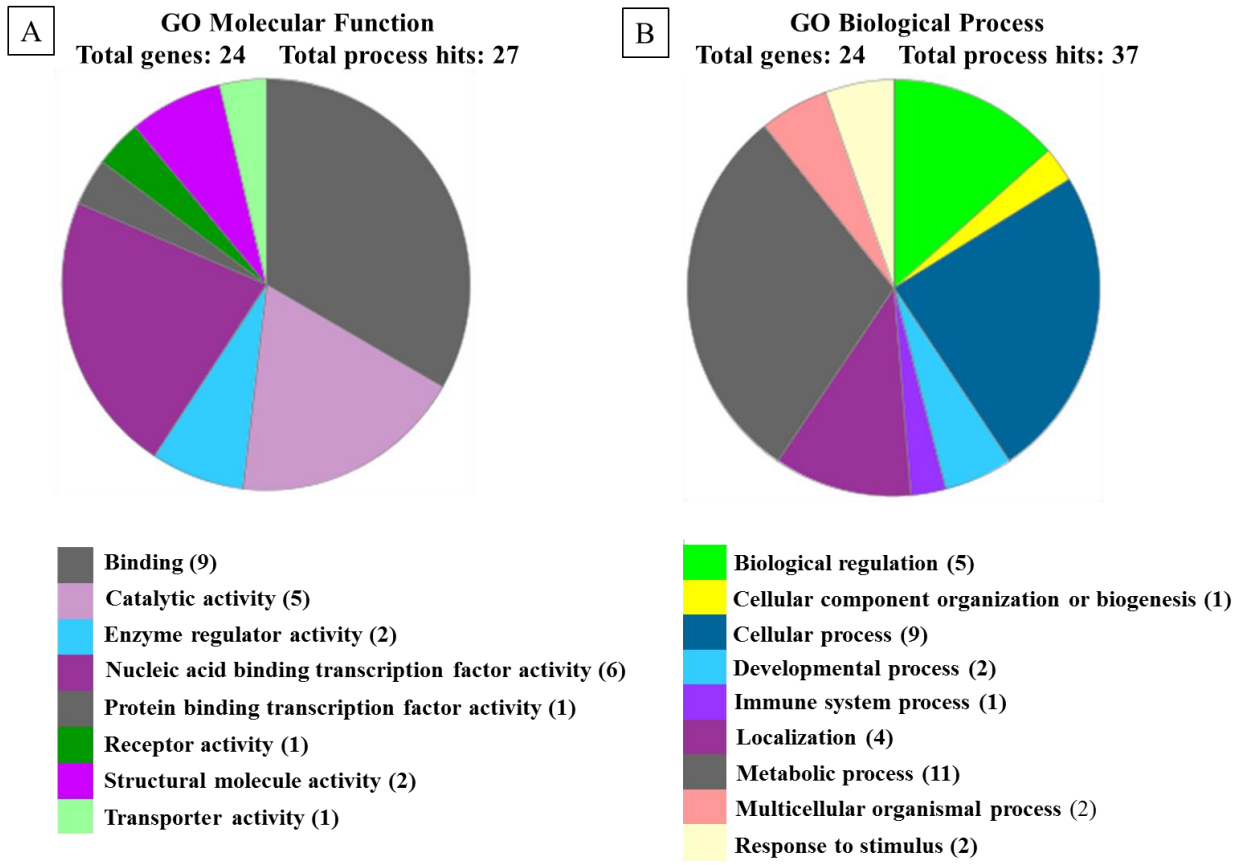

Figure 3. Classification of genes located on chromosomic regions with SNPs moderately associated with clinical pneumonia. Molecular function (A) and biological processes (B) according to PANTHER classification database.

1. Discussion

The accessibility of the equine genome sequence and commercially-available tools for genome-wide screening, such as the EquineSNP70 BeadChip Array (EquineSNP70 BeadChip Array Illumina, San Diego, CA), make it feasible to identify chromosomal regions and candidate genes associated with susceptibility of horses to infectious diseases. The data presented here demonstrated moderate associations of genetic markers with susceptibility of foals to *R. equi* pneumonia. The breeds evaluated here have not been studied with this purpose and approach, further contributing to knowledge gained from previous data from Quarter Horse foals (McQueen et al., 2014; Whitfield-Cargile et al., 2015). Although most GWAS SNP studies use a threshold P value of $\leq 10^{-5}$ for association of a SNP with phenotype, we investigated those SNPs with P value $\leq 10^{-4}$ because of our modest sample size and the high ORs (i.e., estimated magnitude of effect) observed for these SNPs.

The GWAS is an important tool to accesses the genetic variation within different species and breeds; however, some spurious associations may be expected. The SNPs evaluated represent a small proportion of the 2.7 billion DNA base pairs mapped for the horse genome. Additionally, there are several risk factors associated with *R. equi* pneumonia, making it possible for confounding to occur in genetic epidemiological studies. In this study, we sampled foals from different farms, and determinants of risk of *R. equi* pneumonia might have varied by farm. To minimize probable confounding factors, we selected farms endemic to *R. equi* infection, located in the same region of RS state (Brazil), reducing climactic and management variation. Nevertheless, identification of farm as a cause of population stratification (i.e., confounding by farm) in the analysis for comparison 2 highlights the potential for confounding.

As expected, our results do not implicate a single gene or small set of genes as determining susceptibility to *R. equi* pneumonia. Rather, they extend our knowledge of

biological processes and pathways that contribute to susceptibility to this complex disease and suggest a polygenic influence in determining susceptibility to *R. equi* pneumonia. In this study, 78 of 127 SNPs (61%), implicated genes potentially associated with *R. equi* susceptibility (**Table 1**). Fifty-four were found in introns, and 20 were found in intergenic regions. SNPs that are not in protein-coding regions, such as intronic and intergenic SNPs, may still affect gene function such as expression and splicing. For this reason, we searched up-stream and down-stream (± 50 Kb) of each SNP classified as being in an intergenic region to determine whether the SNP might lie in a regulatory region or be a marker of some gene(s). Using this approach, we found sets of genes with functional capability that segregated into chromosomal regions associated with *R. equi* infection.

Several chromosomal regions associated with *R. equi* susceptibility (**Table 2**) were not identified for both breeds studied. Similarly, association of a region on chromosome 11 associated with susceptibility to equine arteritis virus infection varied among breed and was not consistent across breeds (Go et al., 2011). Clustering of genes in specific chromosomal regions that were associated with equine viral arteritis demonstrates how several cellular genes may be related to genetic susceptibility to intracellular pathogens in horses, similar to the results of our study.

A region of chromosome 11 (chr11:43937485-44533143) was positively associated with disease in all comparisons evaluated (combined breeds, BTB only, and BSH only). The region encodes the ABR (active BCR-related) gene, which has been implicated in negative regulation of cellular extravasation, *neutrophil degranulation*, cell migration, *inflammatory responses*, and *phagocytosis* in *Equus caballus* (**Table 3**). Additionally, ABR has been linked to the negative regulation of neutrophil reactive oxygen species (ROS) production (Cunnick et al., 2009) and phagocytosis of opsonized particles during FcγR-mediated phagocytosis in macrophage (Cho et al., 2007). Neutrophil degranulation, inflammatory response, and

phagocytic properties are determinants of *R. equi* infection (Hines et al., 2001). *Rhodococcus equi* lung burdens can be prevented by neutrophils activity, which probably control the bacterium multiplication before adaptive immunity clearance (Martens et al., 2005). At the same time that neutrophil deficiency or inefficiency would contribute to predispose to *R. equi* pneumonia in young foals, an exacerbated neutrophil production might induce pulmonary tissue damage. Pulmonary inflammation, which results in massive neutrophil influx, have been associated to increased susceptibility to *Streptococcus pneumonia* (Bou Ghanem et al., 2015). Whether the positive association between the SNPs found (chromosome 11 region 43937485 to 44533143) with *R. equi* pneumonia is due modulation of ABR gene and consequently impairment of negative regulation of neutrophils influx need to be determined. The genes GNB2L1 (guanine nucleotide-binding protein subunit beta-2-like 1) and FUCA2 (α-L-fucosidase 2) found in a region of chromosome 10 were marked by SNPs significantly associated with *R. equi* pneumonia for comparisons 1 and 2. Both genes have functional activities that may be related to *R. equi* pathogenesis (**Table 2**). GNB2L1 mRNA expression was up-regulated in macrophages infected with *Mycobacterium tuberculosis* (McGarvey et al., 2004), likewise, GNB2L1 was associated with inhibition of *Yersinia pseudotuberculosis* phagocytosis and survival following infection of host cells (http://www.uniprot.org/uniprot/P63244). The FUCA2 gene was found to be essential for *Helicobacter pylori* cell adhesion, pathogenesis, and defense strategy to escape host surveillance (Liu et al., 2009). Once the SNPs nearby GNB2L1 and FUCA2 were positively associated to *R. equi* pneumonia (OR 4.5 – 9) they might modulate the functional activities of these genes (positive regulation of apoptotic process in response to bacterium and regulation of entry of bacterium into host cell, **Table 2**) and to benefit *R. equi* intracellular survival following infection.

The FOXP1 (forkhead box protein P1) gene, found in a region of chromosome 16 where there were several SNPs significantly associated with *R. equi* pneumonia in comparison 2, is associated with many biological functions strongly linked to immune responses against bacterial infection, as summarized in **Table 2**. However, due the lack of scientific data available we were unable to stablish a putative relationship between this gene and predisposition to *R. equi* pneumonia. On the other hand, the TIMP3 gene (which encodes an endogenous metalloprotease inhibitor) implicated in comparison 2 and found in a region of chromosome 28, is involved in cellular processes that are plausible for contributing to susceptibility of foals to *R. equi*. TIMP3 modulation, in response to certain cytokines, leads to different levels of metalloprotease substrates, including ICAM-1 (intracellular adhesion molecule 1) (Esteso et al., 2014). ICAM-1 on alveolar epithelial cells surface promotes mobility of alveolar macrophages (AM) and efficient phagocytosis (Paine et al., 2002). Furthermore, ICAM-1 binds to macrophage adhesion ligand-1 (ITGAM), also known as macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3). Interestingly, we found a SNP (BIEC2_224026, comparison 3) positively associated with *R. equi* infection located in an intronic region of the ITGAM gene. It is well known that *R. equi* requires ITGAM (Mac-1) to bind to mammalian cells (Hondalus et al., 1993). Moreover, the mechanism of *R. equi* ingestion vary with the phagocytic receptor involved and activation of the cell, which appears to influence bacterial clearance. Further investigation of the role of the pathways associated with TIMP3 and ITGAM on susceptibility to *R. equi* pneumonia is required to clarify the meaning of this result.

We were unable to identify significant association with candidate genes previously associated with *R. equi* pneumonia in other horse breeds (Halbert et al., 2006; Horin et al., 2010; McQueen et al., 2014; Mousel et al., 2003). This might be due to differences among study populations (e.g, different breeds) or experimental design (i.e., GWAS versus candidate

gene approach). Conversely, all previous studies have identified genes pertaining to host defenses against infectious pathogens, such as iron transport (Halbert et al., 2006; Mousel et al., 2003) and innate immune responses (Horin et al., 2010; McQueen et al., 2014). Possibly, these apparently discrepant findings might converge around critical biological pathways or processes that influence host defenses against *R. equi* (and other intracellular pathogens).

This study had limitations in addition to those already discussed, including the small sample size and the presence of population stratification. The small sample size was a direct result of the funding available to us for this project and of the costs for genotyping foals. The population stratification appeared to be an effect of farm for the BSH population. Another limitation is that our study classified foals into 2 groups: affected and unaffected foals. It is possible that some of the unaffected foals had subclinical pneumonia. This classification was a result of the fact that none of the farms screened foals for subclinical pneumonia. If the SNPs identified determine infection, then it is possible that we might have missed or underestimated associations because of misclassification of infected foals that were subclinical. We believe, however, that the important clinical distinction is between foals that develop pneumonia and those that do not. In this regard, this limitation can also be considered as a strength of our study. Another strength of our study is that we evaluated 2 horse breeds using GWAS, complementing our previous study of *R. equi* pneumonia in Quarter Horse foals (McQueen et al., 2014).

Despite the limitations of this study, we identified chromosomal regions moderately associated with susceptibility to *R. equi* pneumonia. Our findings further support the results of previous studies documenting the importance of components of innate immunity in protecting against *R. equi* pneumonia (McQueen et al., 2014). Further studies need be performed to determine whether our findings are simply due to linkage disequilibrium or whether they truly represent pathways and processes that impact susceptibility of foals to *R. equi* pneumonia.

Conclusions

The results of this study identify chromosomal regions containing genes involved in innate immune responses to intracellular pathogens, such as neutrophil degranulation, inflammation, and regulation of phagocytosis. Our findings also reinforce the polygenic basis of susceptibility to *R. equi* pneumonia.

Conflict of Interest Declaration

Authors disclose no conflict of interest.

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References

1. Prescott JF (1991) Rhodococcus equi: an animal and human pathogen. Clin Microbiol Rev 4: 20-34.
2. Prescott JF, Meijer WG, Vázquez-Boland JA (2010) Rhodococcus equi. In: Gyles CL, Prescott John F, Songer JG, Thoen, Charles O, editor. Pathogenesis of Bacterial Infections in Animals. 4 ed. Ames: Blackwell Publishing. pp. 149-166.
3. Giguère S, Cohen ND, Chaffin MK, Hines SA, Hondalus MK, et al. (2011) Rhodococcus equi: clinical manifestations, virulence, and immunity. J Vet Intern Med 25: 1221-1230.
4. Venner M, Reinhold B, Beyerbach M, Feige K (2009) Efficacy of azithromycin in preventing pulmonary abscesses in foals. Vet J 179: 301-303.
5. Mousel MR, Harrison L, Donahue JM, Bailey E (2003) Rhodococcus equi and genetic susceptibility: assessing transferrin genotypes from paraffin-embedded tissues. J Vet Diagn Invest 15: 470-472.
6. McQueen CM, Doan R, Dindot SV, Bourquin JR, Zlatev ZZ, et al. (2014) Identification of genomic loci associated with Rhodococcus equi susceptibility in foals. PLoS One 9: e98710.

7. Halbert ND, Cohen ND, Slovis NM, Faircloth J, Martens RJ (2006) Variations in equid SLC11A1 (NRAMP1) genes and associations with Rhodococcus equi pneumonia in horses. J Vet Intern Med 20: 974-979.
8. Horin P, Smola J, Matiasovic J, Vyskocil M, Lukeszova L, et al. (2004) Polymorphisms in equine immune response genes and their associations with infections. Mamm Genome 15: 843-850.
9. McQueen CM, Dindot SV, Foster MJ, Cohen ND (2015) Genetic Susceptibility to Rhodococcus equi. J Vet Intern Med 29: 1648-1659.
10. Yamamoto S, Shimizu S, Kiyonaka S, Takahashi N, Wajima T, et al. (2008) TRPM2-mediated Ca2+influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. Nat Med 14: 738-747.
11. Martens RJ, Cohen ND, Jones SL, Moore TA, Edwards JF (2005) Protective role of neutrophils in mice experimentally infected with Rhodococcus equi. Infect Immun 73: 7040-7042.
12. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81: 559-575.
13. Raudsepp T, McCue ME, Das PJ, Dobson L, Vishnoi M, et al. (2012) Genome-wide association study implicates testis-sperm specific FKBP6 as a susceptibility locus for impaired acrosome reaction in stallions. PLoS Genet 8: e1003139.
14. Wellcome Trust Case Control C (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661-678.
15. Aulchenko YS, Ripke S, Isaacs A, van Duijn CM (2007) GenABEL: an R library for genome-wide association analysis. Bioinformatics 23: 1294-1296.
16. Price AL, Zaitlen NA, Reich D, Patterson N (2010) New approaches to population stratification in genome-wide association studies. Nat Rev Genet 11: 459-463.
17. Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30: 207-210.
18. Whitfield-Cargile CM, Cohen ND, Suchodolski J, Chaffin MK, McQueen CM, et al. (2015) Composition and Diversity of the Fecal Microbiome and Inferred Fecal Metagenome Does Not Predict Subsequent Pneumonia Caused by Rhodococcus equi in Foals. PLoS One 10: e0136586.
19. Go YY, Bailey E, Cook DG, Coleman SJ, Macleod JN, et al. (2011) Genome-wide association study among four horse breeds identifies a common haplotype associated with in vitro CD3+ T cell susceptibility/resistance to equine arteritis virus infection. J Virol 85: 13174-13184.
20. McGarvey JA, Wagner D, Bermudez LE (2004) Differential gene expression in mononuclear phagocytes infected with pathogenic and non-pathogenic mycobacteria. Clin Exp Immunol 136: 490-500.
21. Liu TW, Ho CW, Huang HH, Chang SM, Popat SD, et al. (2009) Role for alpha-L-fucosidase in the control of Helicobacter pylori-infected gastric cancer cells. Proc Natl Acad Sci U S A 106: 14581-14586.
22. Cunnick JM, Schmidhuber S, Chen G, Yu M, Yi SJ, et al. (2009) Bcr and Abr cooperate in negatively regulating acute inflammatory responses. Mol Cell Biol 29: 5742-5750.
23. Cho YJ, Cunnick JM, Yi SJ, Kaartinen V, Groffen J, et al. (2007) Abr and Bcr, two homologous Rac GTPase-activating proteins, control multiple cellular functions of murine macrophages. Mol Cell Biol 27: 899-911.
24. Hines MT, Paasch KM, Alperin DC, Palmer GH, Westhoff NC, et al. (2001) Immunity to Rhodococcus equi: antigen-specific recall responses in the lungs of adult horses. Vet Immunol Immunopathol 79: 101-114.

25. Esteso G, Luzon E, Sarmiento E, Gomez-Caro R, Steinle A, et al. (2014) Altered microRNA expression after infection with human cytomegalovirus leads to TIMP3 downregulation and increased shedding of metalloprotease substrates, including MICA. J Immunol 193: 1344-1352.
26. Paine R, 3rd, Morris SB, Jin H, Baleeiro CE, Wilcoxen SE (2002) ICAM-1 facilitates alveolar macrophage phagocytic activity through effects on migration over the AEC surface. Am J Physiol Lung Cell Mol Physiol 283: L180-187.
27. Hondalus MK, Diamond MS, Rosenthal LA, Springer TA, Mosser DM (1993) The intracellular bacterium Rhodococcus equi requires Mac-1 to bind to mammalian cells. Infect Immun 61: 2919-2929.
28. Horin P, Sabakova K, Futas J, Vychodilova L, Necesankova M (2010) Immunity-related gene single nucleotide polymorphisms associated with Rhodococcus equi infection in foals. Int J Immunogenet 37: 67-71.

5. CONSIDERAÇÕES FINAIS

Embora R. equi tenha sido descoberto em 1923, e a partir deste momento grupos de pesquisa no mundo inteiro estejam buscando conhecer e controlar a infecção por R. equi, está é ainda uma enfermidade que ocorre em diversos países, tanto desenvolvidos quanto subdesenvolvidos. No Brasil, a rodococose equina cursa com elevada mortalidade e significativas perdas econômicas. Além dos desafios relacionados ao controle e prevenção da doença, existe uma crescente preocupação em relação à resistência antimicrobiana. Somando-se às falhas no tratamento, a inexistência de uma vacina disponível comercialmente torna o controle da doença ainda mais difícil.

Rhodococcus equi é um patógeno extremamente versátil, uma vez que transita por um estilo de vida saprófito e patogênico. A intensa relação de R. equi com o hospedeiro, destacando-se os equinos jovens, parece ser um fator determinante para a evolução da bactéria, a qual tem demonstrado um variado repertório de mecanismos de virulência e evasão do sistema imune. Consequentemente, R. equi tornou-se uma importante fonte de estudos não apenas devido a sua elevada patogenicidade para equinos, mas também como um exemplo de micro-organismo que evoluiu e evolui de forma coordenada com seu hospedeiro.

Diante das inúmeras características da patogenia da rodococose equina, a sobrevivência intracelular de isolados virulentos de R. equi possui destacada importância. Embora intensamente estudada, a capacidade de R. equi replicar no interior de macrófagos é fonte para muitas hipóteses científicas. Uma vez que se torna impossível abordar todos os mecanismos potencialmente associados à esta característica, nosso grupo de estudo buscou entender a importância do Fe no sucesso de R. equi em ambiente intracelular. Além disso, buscou-se investigar a susceptibilidade genética associada a rodococose equina, salientando a importância de polimorfismos no gene da Tf como possíveis fatores de risco.

Com base em nossos resultados, ressaltamos o papel indispensável do Fe, especialmente durante a multiplicação de R. equi no interior de macrófagos. Demonstramos que a sobrevivência deste patógeno pode ser inibida através de drogas cujo mecanismo de ação está relacionado à modulação, neste caso à diminuição, da disponibilidade de Fe intracelular.

Ainda, nossos resultados apontam para a existência de susceptibilidade genética associada à rodococose em potros. Observou-se diversos genes positivamente associados à ocorrência de pneumonia por R. equi, corroborando com estudos prévios, os quais tem sugerido uma influência poligênica na infecção por este patógeno. Destacamos ainda, o

potencial de diferentes variantes de Tf equina, especialmente as variantes D e F, na predisposição à doença. Por fim, espera-se que os resultados aqui apresentados possam representar subsídios para o entendimento e controle desta importante enfermidade que compromete a indústria equina.

6. CONCLUSÕES

- *Rhodococcus equi* está evoluindo de forma a especializar-se na aquisição e utilização de Fe a partir do hospedeiro.
- A aquisição e utilização de Fe por *R. equi* devem ser considerados pontos chave no desenvolvimento de agentes quimioterápicos.
- Uma vez que *R. equi* codifica redundantes mecanismos de aquisição e utilização de Fe, é provável que agentes quimioterápicos deverão inibir múltiplos mecanismos ou ser utilizados em combinação.
- O conceito de "imunidade nutricional" pode considerado uma importante estratégia para minimizar a resistência antimicrobiana observada em *R. equi.*
- Chloroquine é capaz de inibir a multiplicação intracelular de *R. equi*, muito provavelmente à deprivação de Fe intracelular.
- São necessários estudos avaliando o potencial terapêutico de chloroquine como tratamento alternativo de infecções por *R. equi.*
- Existe importantes regiões cromossômicas positivamente associadas à pneumonia por *R. equi*, as quais parecem possuir genes associados à resposta imune contra patógenos intracelulares.
- Como postulado por estudos anteriores, classificamos a rodococose equina como uma enfermidade de base poligênica.
- Polimorfismos no gene da Tf ocorrem em equinos da raça BSH e BTB, inclusive polimorfismos ainda não descritos na literatura.
- Existe a ocorrência de dois alelos entre as raças estudas, incluindo animais heterozigotos para estes alelos.
- Acredita-se que exista uma relação entre variantes de Tf equina e susceptibilidade genética à pneumonia por *R. equi* nas raças analisadas.

REFERÊNCIAS BIBLIOGRÁFICAS

AINSWORTH, D. M. et al. Athletic performance of horses previously infected with *R. equi* pneumonia as foals. In: **Proceedings of the 43rd Anual Convention of the American Association of Equine Practitioners (AAEP)**, Denver, v. 43, p. 81-82, 1997.

AISEN, P.; LISTOWSKY, I. Iron transport and storage proteins. **Annual Review of Biochemistry**, v. 49, p. 357-393, 1980.

ANDREWS, S. C. et al. Bacterial iron homeostasis. **FEMS Microbiology Reviews**, v. 27, p. 215-237, 2003.

BALTES, N.; HENNIG-PAUKA, I.; GERLACH, G. F. Both transferrin binding proteins are virulence factors in *Actinobacillus pleuropneumoniae* serotype 7 Infection. **FEMS Microbiology Letters**, v. 209, p. 283-287, 2002.

BELL, K. et al. Subdivision of equine TF into H1 and H2. **Animal Genetic**, v. 19, p. 177-183, 1988.

BENOIT, S. et al. H2O2, which causes macrophage-related stress, triggers induction of expression of virulence-associated plasmid determinants in *Rhodococcusequi*. **Infection and Immunity**, v. 70, p. 3768-3776, 2002.

BRAEND, M.; STORMONT, C. Studies on haemoglobin and transferrin types of horses. **Nordisk Veterinaermedicin**, v. 16, p. 31-37, 1964.

BRANDON, R. B.; GIFFARD, J. M.; BELL, K. Single nucleotide polymorphisms in the equine transferrin gene. **Animal Genetics**, v. 30, p. 439-443, 1999.

BRAUM, V. Iron uptake mechanisms and their regulation in pathogenic bacteria. **International Journal of Medical Microbiology,** v. 291, p. 67-79, 2001.

BUNTAIN, S. et al. Frequency of *Rhodococcus equi* in Feces of Mares in Central Kentucky. J**ournal of Equine Veterinary Science, v.** 30, n. 4, p. 191-195, 2010.

BUCKLEY, T. E.; STANBRIDGE, S. Resistance studies of erythromycin and rifampin for *Rhodococcus equi* over a 10-year period. **Irish Veterinary Journal**, v. 60, p. 728-731, 2007.

BYRNE, B. A. et al. Virulence plasmid of *Rhodococcus equi* contains inducible gene family encoding secreted proteins. **Infections and Immunity**, v. 69, n. 2, p. 650-656, 2001.

BYRD, T. F.; HORWITZ, M. A. Aberrantly low transferrin receptor expression on human monocytes is associated with nonpermissiveness for *Legionella pneumophila* growth. **Journal of Infectious Disease**, v. 181, p. 1394-1400, 2000.

CEPEA. **Centro de estudos avançados em economia aplicada: Estudo do complexo agronegócio cavalo.** Piracicaba, junho de 2006.

CHAFFIN, M. K.; COHEN, N. D.; MARTENS, R. J. Evaluation of equine breeding farm characteristics as risk factors for development of *Rhodococcus equi* pneumonia in foals. **Journal of the American Veterinary Medical Association**, v. 222, p. 467-475, 2003.

CHUNG, M. C. M.; McKENZIE, H. A. Studies on equine transferrin-I: The isolations and partial characterization of the D and R variants. **Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology**, v. 80, p. 287-297, 1985.

COHEN, N. D. et al. Farm characteristics and management practices associated with development of *Rhodococcus equi* pneumonia in foals. **Journal of American Veterinary Medical Association**, v. 226, n. 3, p. 404-413, 2005.

COHEN, N.D. et al. Association of soil concentration of *Rhodococcus equi* and incidence of pneumonia attributable to *Rhodococcus equi* in foal on farms in central of Kentucky. **American Journal of Veterinary Research**, v. 69, n. 3, p. 1-11, 2008.

COHEN, N. D. et al. Virulent *Rhodococcus equi* with location (stall versus paddock) and month (January through June) on 30 horse breeding farms in central Kentucky. **American Journal of Veterinary Research**, v. 73, n. 10, p. 1603-1609, 2012.

COHEN, N. D. et al. Association of perinatal exposure to airborne *Rhodococcus equi* with risk of pneumonia caused by *R equi* in foals. **American Journal of Veterinary Research**, v. 74, n. 1, p. 102-109, 2013.

De JONG, G.; Van DIJK, J. P.; Van EIJK, H. G. The biology of transferrin. **Clinica Chimica Acta**, v. 190, p. 1-46, 1990.

DEPRÁ, N. M. et al. Monitoramento da infecção por *Rhodococcus equi* em potros puro sangue de corrida. **Arquivo Faculdade Veterinária-UFRGS**, v. 29, p. 25-35, 2001.

De VOSS, J. J. et al. The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. **Proceedings of the National Academy of Sciences of the United States of America**, v. 97, p. 1252-1257, 2000.

DIAS, I. M. G. et al. Formação e estrutura populacional do eqüino Brasileiro de Hipismo. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 52, n. 6, 2000.

DRECHSEL, H.; WINKELMANN, G. Iron chelation and siderophores. In: Winkelmann, G.; Carrano, C. J. (Orgs.). **Transition metals in microbial metabolism**. Harwood Academic Publishers, Amsterdam, 1997, p. 1-49.

FAO - Food and Agriculture Organization. Online. Disponível em http://ftp.fao.org/agl/agll/docs/fertusebrazil.pdf. Acesso em 13 outubro de 2014

FARINA, C. et al. *Rhodococcus equi* infection in non-HIV-infected patients: two case reports and review. **Clinical Microbiology and Infection**, v. 3, n. 1, p. 12-18, 1997.

FINNERTY, W. R. The biology and genetics of the genus Rhodococcus. **Annual Review of Microbiology**, v. 46, p. 193-218, 1992.

GYLES, C. L. et al. **Pathogenesis of bacterial infections in animal**. *Rhodococcus*. 4. ed. 2010.

GUIGUÈRE, S. *Rhodococcus equi* pneumonia. In: **Proceedings 47th Annual Meeting American Association of Equine Practitioners**, San Diego, v. 47, p. 456-467, 2001.

GIGUÈRE, N. D. et al. Diagnosis, treatment, control, and prevention of infections caused by *Rhodococcus equi* in foals. **Journal of Veterinary Internal Medicine**, v. 25, p. 1209-1220, 2011.

GOLUB, B.; FALK, G.; SPINK, W. W. Lung abscess due to *Corynebacterium equi*. Report of first human infection. **Annals of Internal Medicine**, v. 66, n. 6, p. 1174-1177, 1967.

GOTTSCHALK, R. et al. Total iron-binding capacity and serum transferrin determination under the influence of several clinical conditions**. Clinica Chimica Acta**, v. 293, n. 1-2, p. 127-138, 2000.

GRAY-OWEN, S. D.; SCHRYVERS, A. B. Characterization of transferrin binding proteins 1 and 2 in invasive type b and nontypeable strains of *Haemophilus influenzae*. **Infection and Immunity**, v. 63, p. 3809-3815, 1995.

HALBERT, N. D. et al. Variations in equid SLC11A1 (NRAMP1) genes and associations with *Rhodococcus equi* pneumonia in horses. **Journal of Veterinary Internal Medicine**, v. 20, p. 974-979, 2006.

HONDALUS, M. K.; MOSSER, D. M. Survival and peplication of *Rhodococcus equi* in macrophages. **Infection and immunity**, v. 62, n. 10, p. 4167-4175, 1994.

HORIN, P. et al. Polymorphisms in equine immune response genes and their associations with infections. **Mammalian Genome**, v. 15, n. 10, p. 843-850, 2004.

HORIN, P. et al. Single nucleotide polymorphisms of interleukin-1 beta related genes and their association with infection in the horse. **Developments in Biological**, v. 132, p. 347-351, 2008.

HORIN, P. et al. Immunity-related gene single nucleotide polymorphisms associated with *Rhodococcus equi* infection in foals. **Internation Journal of Immunogenetics**, n. 37, p. 67-71, 2009.

JORDAN, M. C. et al. Effects of iron modulation on growth and viability of *Rhodococcus equi* and expression of virulence-associated protein A. **American Journal of Veterinary Research**, v. 64, n. 11, p. 1337-1346, 2003.

JUKES, T. H.; CANTOR, C. R. Evolution of protein molecules. In: MUNRO, H. N. (Org.). **Mammalian Protein Metabolism**. Academic Press, New York, 1969, p. 21-132.

KAMINSKI, M. et al. Polymorphism of transferrin locus in horses: immunochemical evidence of two structural different subgroups of allelic proteins. **Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology,** v. 68, p. 505-507, 1981.

KASVOSVE, I. et al. Transferrin polymorphism influences iron status in blacks. **Clinical Chemistry**, v. 46, n. 10, p. 1535-1539, 2000.

LEBLANC, M. M. et al. Factors that influence passive transfer of immunoglobulins in foals. **Journal of American Veterinary Medical Association**, v. 200, p. 179-183, 1992.

LETEK, M. et al. The genome of a pathogenic *Rhodococcus*: cooptive virulence underpinned by key gene acquisitions. **PLoS Genetics**, 6(9), e1001145, 2010.

MACGOWAN, K. L.; MANGANO, M. F. Infections on *Rhodococcus equi* in children. **Diagnostic Microbiology and Infectious Disease,** v. 14, p. 347-352, 1991.

McQUEEN, C. M. et al. Identification of genomic loci associated with *Rhodococcus equi* susceptibility in foals**. PLoS ONE**, 9(6), e98710, 2014.

MIRANDA-CASOLUENGO, R. et al. The intracellular pathogen *Rhodococcus equi* produces a catecholate siderophore required for saprophytic growth. **Journal of Bacteriology**, v. 190, n. 5, p. 1631-1637, 2008.

MIRANDA-CASOLUENGO, R. et al. The hydroxamate siderophore rhequichelin is required for virulence of the pathogenic actinomycete *Rhodococcus equi*. **Infection and Immunity**, v. 80, n. 12, p. 4106-4114, 2012.

MOUSEL, M. R. et al. *Rhodococcus equi* and genetic susceptibility: assessing transferrin genotypes from paraffin-embedded tissues. **Journal of Veterinary Diagnostic Investigation**, v. 15, p. 470-472, 2003.

MUSCATELLO, G. et al. *Rhodococcus equi* infection in foals: the science of 'rattles'. **Equine Veterinary Jorunal,** v. 39, p. 470-478, 2007.

MUSCATELLO, G. *Rhodococcus equi* pneumonia in the foal – Part 2: diagnostics, treatment and disease management. **The Veterinary Journal,** v. 192, p. 27-33, 2012.

NIWA, H.; HOBO, S.; ANZAI, T. A nucleotide mutation associated with fluoroquinolone resistance observed in gyrA of in vitro obtained *Rhodococcus equi* mutants. **Veterinary Microbiology,** v. 115, p. 264-268, 2006.

NEWTON, J. R. et al. Evidence for transferrin allele as a host-level risk factor in naturally occurring equine respiratory disease: a preliminary study. **Equine Veterinary Journal**, v. 39, n. 2, p. 164-171, 2007.

PRESCOTT, J. F. *Rhodococcus equi*: an animal and human pathogen. **Clinical Microbiology Reviews**, v. 4, n.1, p. 20-34, 1991.

PUTNAM, F. W.; ROOP, W. E. Purification and characterization of genetic variants of human transferrins. **Bibliotheca Haematologica**, v. 17, p. 231178-231180, 1965.

RATLEDGE, C.; DOVER, L. G. Iron metabolism in pathogenic bacteria. **Annual Review of Microbiology**, v. 54, p. 881-941, 2000.

RIBEIRO, M. G. et al. Molecular epidemiology of virulent *Rhodococcus equi* from foals in Brazil: virulence plasmids of 85-kb type I, 87-kb type I, and a new variant, 87-kb type III. **Comparative Immunology, Microbiology & Infectious Diseases**, v. 28, p. 53-61, 2005.

RUSELL, D. A. et al. The LysR-Type Transcriptional Regulator VirR Is Required for Expression of the Virulence Gene *vapA* of *Rhodococcus equi* ATCC 33701. **Journal of Bacteriology**, v. 186, n. 17, p. 5576-5584, 2004.

SELLON, D. C. Investigating outbreaks of respiratory disease in older foals. **In: Proceedings of the 47th Anual Convention of the American Association of Equine Practitioners**, San Diego, v. 47, p. 447-455, 2001.

SMITHIES, O. Variations in serum b-globulins. **Nature**, v. 180, p. 1482-1483, 1957.

SPOONER, R. L. et al. Isolation and partial characterization of sheep transferrin. **Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology**, v. 52, p. 515-522, 1975.

TAMURA, K. et al. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. **Molecular Biology and Evolution**, v. 30, p. 2725-2729, 2013.

TAN, C. et al. Molecular characterization of a lipid-modified virulence-associated protein of *Rhodococcus equi* and its potential in protective immunity. **Canadian Journal of Veterinary Research,** v. 59, p. 51-59, 1995.

TAKAI, S. et al. Quantitative aspects of fecal *Rhodococcus (Corynebacterium) equi* in foals. **Journal of Clinical Microbiology**, v. 23, p. 794-796, 1986.

TAKAI, S.; SEKISAKI, T.; OSAWA, A. Association between a large plasmid and 15-17 kDa antigens in virulent *Rhodococcus equi*. **Infection and Immunity**, v. 59, p. 4056-4060, 1991.

TAKAI, S. et al. Virulence-associated plasmids in *Rhodococcus equi*. **Journal of Clinical Microbiology**, v. 31, p. 1726-1729, 1993.

TAKAI, S. et al. Identification of virulent *Rhodococcus equi* by amplification of gene coding 15-to 17-Kilodalton antigens. **Journal of Clinical Microbiology**, v. 33, p. 1624-1627, 1995.
TAKAI, S. et al. Isolation of virulent and intermediately virulent *R. equi* from soil and sand on parks and yards in Japan. **Journal of Veterinary Medicine Science**, v. 58, n. 7, p. 669-672, 1996.

TAKAI, S. et al. Isolation of virulent and intermediately virulent *Rhodococcus equi* from soil and sand on parks and yards in Japan. **Journal of Veterinary Medicine Science**, v. 58, n. 7, p. 669-672, 1997.

THOMPSON, J. D. et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. **Nucleic Acids Research**, v. 25, p. 4876-4882, 1997.

TOYOOKA, K.; TAKAI, S.; KIRIKAE, T. *Rhodococcus equi* can survive a phagolysosomal environment in macrophages by suppressing acidification of the phagolysosome. **Journal of Medical Microbiology**, v. 54, p. 1007-1015, 2005.

VENNER, M. et al. Failure of antimicrobial therapy to accelerate spontaneous healing of subclinical pulmonary abscesses on a farm with endemic infections caused by *Rhodococcus equi*. **The Veterinary Journal**, v. 192, p. 293-298, 2012.

WADA, R. et al. Pathogenicity and virulence of *Rhodococcus equi* in foals following intratracheal challenge. **Veterinary Microbiol,** v. 56, n. 3-4, p. 301-312, 1997.

APÊNDICE A – TRANSFERRIN GENE POLYMORPHISMS AND ASSOCIATION WITH GENETIC SUSCEPTIBILITY TO *Rhodococcus equi* FOAL PNEUMONIA

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(Artigo **em preparação** para publicação - Veterinary Microbiology)

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1. Introduction

The equine rhodococcosis is among the major causes of death in foals worldwide (Muscatello et al., 2007). This disease is caused by *Rhodococcus equi*, an gram-positive intracellular facultative pathogen that replicates within macrophages, triggering severe respiratory disorder in foals, which are infected shortly after birth (Hondalus and Mosser, 1994). Due to lack of effective methods of control and prevention of the disease, the animals are monitored under stringent management, which often are not enough to prevent outbreaks, especially in endemic farms. Several factors have been associated with increased risk of *R. equi* pneumonia, as farm large acreage, transient population, high foal density (Chaffin et al., 2003), and early exposure of foals to airborne virulent *R. equi* (Cohen et al., 2013). Studies addressed foal-related risk factors are scarce and it is not known why some foals develop *R. equi* pneumonia and others do not, even under relatively same conditions. Nevertheless, host-immune-related (IR) markers have been associated with variation in the shedding status of *R. equi* in foals (Horin et al., 2008; Horin et al., 2010; Horin et al., 2004; McQueen et al., 2014; Mousel et al., 2003).

Recently an important study performed independent single nucleotide polymorphism (SNP) in Quarter Horse breed and verified the transient receptor potential cation channel (*TRMP2*) gene, related to innate immune response, as potential candidate gene associated with *R. equi* pneumonia (McQueen et al., 2014). Other genes associated to iron transport and immune defense against infectious pathogens, such as Tf (transferrin) (Mousel et al., 2003) and *SLC11A1* (solute carrier family 11, member 1) (Halbert et al., 2006), have been also linked to *R. equi* genetic susceptibility in foals.

Transferrin (Tf) is an iron-binding plasma protein that occurs in a range of variants/alleles in mammals (Brandon et al., 1999; Carpenter and Broad, 1993a). Equine Tf consists of 24 exons located to chromosome 16 according to Ensemble data base (http://useast.ensembl.org/). Fifteen Tf variants were described by biochemical typing and the most common are D, F1, F2, H2, O and R; however F1/F2, D/H2 and O/R are not distinguish by the molecular typing (Brandon et al., 1999). Likewise in humans (Barber and Elde, 2014), the polymorphisms in the horse Tf gene have been studied because their relation with variants (alleles) possibly associated to genetic susceptibility to infectious disease. In this way, (Mousel et al., 2003) and (Newton et al., 2007) suggested that Tf variants are associated to susceptibility or resistance/tolerance to *R. equi* foal pneumonia. According to this authors, there are evidences that possession of D allele is protective and F allele is associated with increased risk.

Once *R. equi* codify redundant mechanisms to access Fe from the host (Letek et al., 2010) and its ability to acquire Fe from the host was considered a determining for intracellular survival (Miranda-CasoLuengo et al., 2012), we aimed herein to better understanding the relationship between Tf variants and *R. equi* genetic susceptibility, as well as to verified the previous findings with D and F allele in a different horse breed not evaluated yet.

2. Materials and Methods

2.1 Ethical statement

All procedures for this study were assessed and approved by the Ethics Committee of Animal Use (Protocol 133/2014), Federal University of Santa Maria, Brazil, were this study was performed. A document attesting the consent to participation in the study providing samples as well as access to animals and data historic was assigned by animal owners.

2.2 Study population

The study population was comprised of 76 horses, 47 Brazilian Sport Horses (BSH) and 29 Brazilian Thoroughbred (BTB) from Rio Grande do Sul, Brazil. The horses belonged to farms with rhodococcosis historic and veterinarians with expertise in *R. equi* infection diagnosis. The animals with historic of *R. equi* infection were diagnosed by clinical signs suggestive of pneumonia (fever, lethargy, cough, nasal discharge, polysynovitis, tachypnea, increased respiratory effort, respiratory distress, and pulmonary crackles or wheezes via thoracic auscultation) and thoracic ultrasonography. Foals that showed no clinical signs of pneumonia as well as ultrasonography evidence of pulmonary consolidation or abscessation, were classified as unaffected.

2.3 Samples and genomic DNA isolation

Venous blood was collected by jugular venipuncture in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing EDTA. Genomic DNA was isolated from whole blood leucocytes by DNeasy® Blood & Tissue Kit (Qiagen®) according to the manufacturer's protocol and stored at -20° C until use. DNA samples with A_{260}/A_{280} ratio between 1.7 and 1.9 were used.

2.4 DNA amplification

We selected the exons 13, 14, 15, and 16 of Tf gene for sequencing in this study. These exons corresponded to exons 12 (cDNA 1466 – 1610 bp), 13 (cDNA 1611 – 1675 bp), 14 (cDNA 1676 – 1860 bp), and 15 (cDNA 1865 – 2050 bp), according to Ensemble data base. All exons are located into the cDNA transcript correspondent to the domain 2 of Tf gene, which codify the C-lobe of the Tf protein (**Figure 1**).

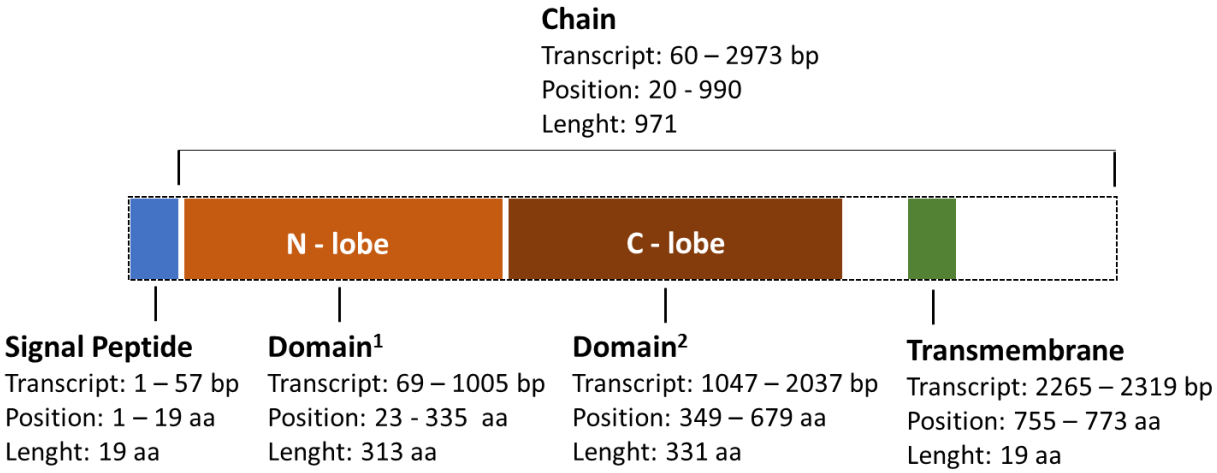


Figure 2. Schematic diagram of Tf gene according to information available in Ensemble data base (http://www.ensembl.org/index.html).

The primers used (**Table 1**) were designed using Primer3 software and synthesized by Sigma-Aldrich (Brazil). PCR reactions were 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. 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 at 30 sec, annealing temperature at 30 sec, extension of 72 °C at 1 min 30 sec followed

by a final extension of 72 °C at 5 min. PCR amplicons were resolved on a 1% agarose gel and photographed by L-PIX ST (Loccus® Brazil).

Table 1. Primers sequences and annealing temperatures used for PCR amplification of exons 13, 14, 15, and 16 from Tf gene.

Exon	Primers (forward/reverse)	Size of PCR product (bp)	Annealing temperature (ºC)
13	5'-CTGGCAGGTCCCGAGTTG-3' 5'-CGCAGGACTAACAGCCTAGG- 3'	400	66
14	5'-CCCTCCTGAATGGCAAATGT-3' 5'-AAGGCATCCTGAACACAAGTGA-3'	200	65
15	5'-TGAGTTTCGGCTTCTCCACATA-3' 5'-GCCTAGGAGATCCGGTTCCA-3'	500	67
16	5'-CTGAGGCATCTTCTGCAGCTT-3' 5'-CCACCTTATCATCTGCCCATACA-3'	500	66

2.5 Nucleotide sequencing and polymorphism analysis

For nucleotide sequencing, 30–60 ng of DNA amplicon, 4.5 pmol of forward or reverse primers (described in the Table 1), and ultrapure water to a final volume of 6 µl were used. Sequencing reactions were performed in duplicates (each product was sequenced twice in both the directions) by ACTGene Analises Moleculares Ltd. (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer armed with 50-cm capillaries and POP6 polymer (Applied Biosystems, Carlsbad, CA, USA). The quality of the obtained DNA sequences was analyzed by the GAP software implemented in the Staden Package (Staden, 1996) and the over lapping fragments of each sequence were assembled. The consensus sequences of each exon were aligned and edited and submitted for comparison with the respective sequences deposited in the GenBank by MEGA 6.0 software (Tamura et al., 2007) and the polymorphic sites were visualized.

3. Results

3.1 Polymorphisms in exons 13, 14, 15, and 16

Sequencing the exons 13, 14, 15, and 16 (named according to GenBank) we were able to cover 79.2% (n = 19) from the total SNPs described in **Table 2** (n = 24). The nucleotide sequence alignment of the exons with all Tf variants previously described (Carpenter and Broad, 1993b, 1994) (**Figure 2**) allowed us to visualize the polymorphisms present in our products.

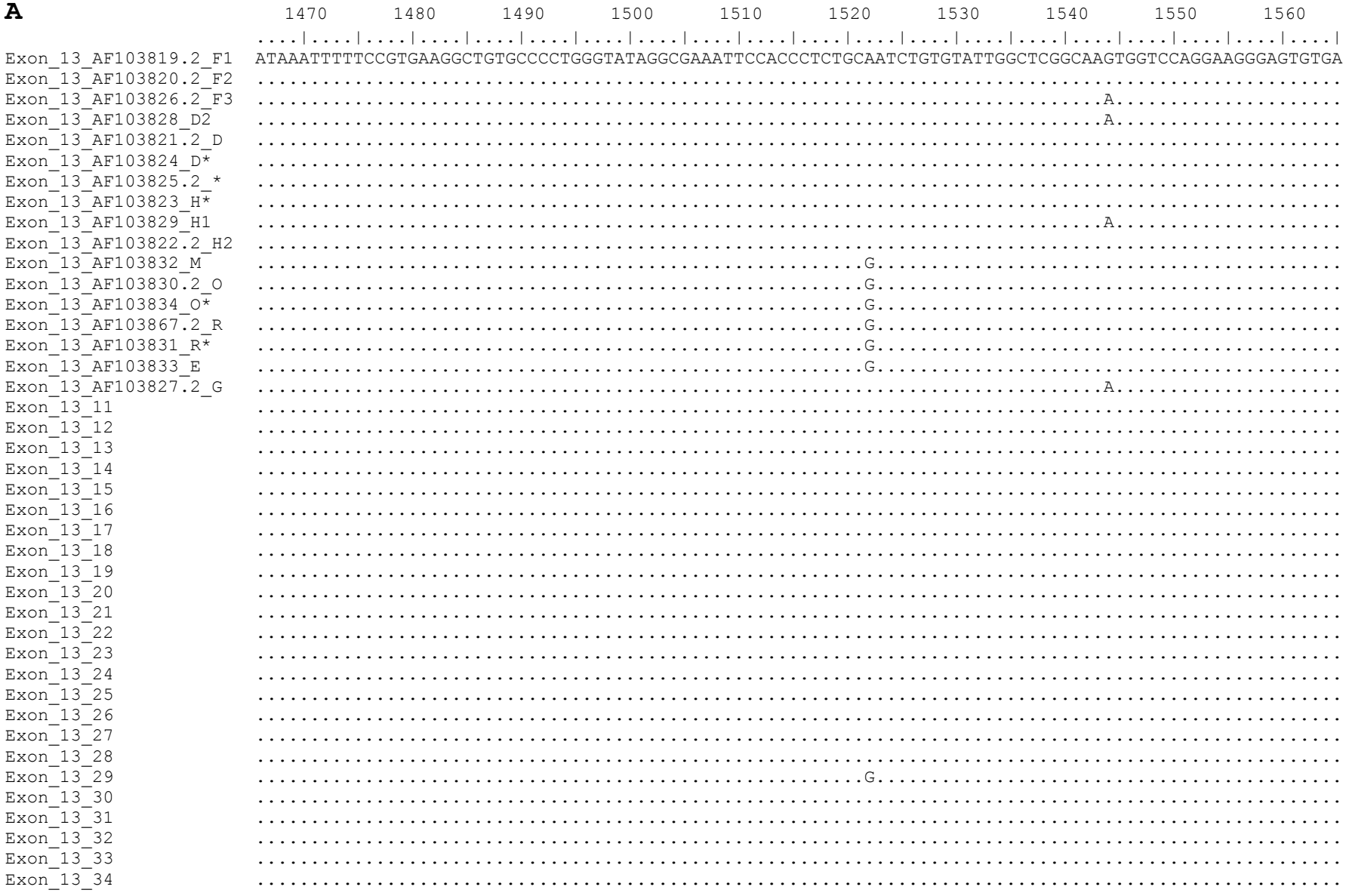
Table 2. Polymorphisms described in the Tf gene.

Exon GB/Ensemble	SNP	SNP code	cDNA position	Protein aa	Codon	aa code	aa name	SNP class
4/3	G/C	S	264	88	CTG, CTC	L/L	Leucine	Synonymous
6/5	G/A	R	573	191	CCG, CCA	P/P	Proline	Synonymous
10/9	T/C	Y	1143	381	ACT, ACC	T/T	Threonine	Synonymous
11/10	A/C	M	1252	418	AAC, CAC	N/H	Asparagine/Histidine	Missense
13/12	G/A	R	1522	508	GAT, AAT	D/N	Aspartic acid/Asparagine	Missense
14/13	G/A	R	1660	554	GAA, AAA	E/L	Glutamic acid/Leucine	Missense
15/14	**C/T**	**Y**	**1678**	**560**	**CGT, TGT**	**R/C**	**Arginine/Cysteine**	**Missense, Splice region**
15/14	G/A	R	1690	564	GAT, AAT	D/N	Aspartic acid/Asparagine	Missense
15/14	G/A	R	1715	572	GGA, GAA	G/E	Glycine/Glutamic acid	Missense
15/14	C/A	M	1723	575	CAG, AAG	Q/K	Glutamine/Lysine	Missence
15/14	C/T	Y	1734	578	TGC, TGT	C/C	Cysteine	Synonymous
15/14	G/T	K	1753	585	GCT, TCT	A/S	Alanine/Serine	Missense
15/14	T/C	Y	1767	589	TTT, TTC	F/F	Phenylalanine	Synonymous
15/14	**G/A**	**R**	**1768**	**590**	**GAG, AAG**	**E/K**	**Glutamic acid/Lysine**	**Missense**
15/14	G/A	R	1770	590	GAG, GAA	E/E	Glutamic acid	Synonymous
15/14	A/T	W	1777	593	AAC, TAC	N/Y	Asparagine/Tyrosine	Missense
15/14	A/C	M	1786	596	AAA, CAA	K/Q	Lysine/Glutamine	Missense
15/14	A/G	R	1786	596	AAA, AGA	K/R	Lysine/Arginine	Missense
15/14	A/G	R	1787	596	AAA, AA-	K/X	Lysine/-	Frameshift
15/14	C/T	Y	1837	613	CGC, TCG	R/C	Arginine/Cysteine	Missense
15/14	G/C	S	1840	614	GAA, CAA	E/Q	Glutamic acid/Glutamine	Missense

15/14	A/G	R	1842	614	GAA, GAG	E/E	Glutamic acid	Synonymous
15/14	G/A	R	1850	617	CGC, CAC	R/H	Arginine/Histidine	Missense
21/-	T/C	Y	2478	826	AAT, AAC	N/N	Asparagine	Synonymous

Source: Ensemble data base (http://www.ensembl.org/index.html).

A


1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
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Exon_13_AF103820.2_F2
Exon_13_AF103826.2_F3
Exon_13_AF103828_D2
Exon_13_AF103821.2_D
Exon_13_AF103824_D*
Exon_13_AF103825.2_*
Exon_13_AF103823_H*
Exon_13_AF103829_H1
Exon_13_AF103822.2_H2
Exon_13_AF103832_M
Exon_13_AF103830.2_O
Exon_13_AF103834_O*
Exon_13_AF103867.2_R
Exon_13_AF103831_R*
Exon_13_AF103833_E
Exon_13_AF103827.2_G
Exon_13_11
Exon_13_12
Exon_13_13
Exon_13_14
Exon_13_15
Exon_13_16
Exon_13_17
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Exon_13_34

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Exon_13_35            ...............................................................................................
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Exon_13_143           ...............................................................................................
Exon_13_144           ...............................................................................................
Exon_13_145           ...............................................................................................
Exon_13_146           ..............................................................................R................
Exon_13_147           ...............................................................................................
Exon_13_148           ...............................................................................................

                          1570      1580      1590      1600      1610
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Exon_13_AF103820.2_F2  ........................................
Exon_13_AF103826.2_F3  ........................................
Exon_13_AF103828_D2    ........................................
Exon_13_AF103821.2_D   ........................................
Exon_13_AF103824_D*    ........................................
Exon_13_AF103825.2_*   ........................................
Exon_13_AF103823_H*    ........................................
Exon_13_AF103829_H1    ........................................
Exon_13_AF103822.2_H2  ........................................
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Exon_13_AF103831_R*    ........................................
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Exon_13_AF103827.2_G   ........................................
Exon_13_11             ........................................
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Exon_13_12          ........................................
Exon_13_13          ............................Y...........
Exon_13_14          ........................................
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Exon_13_144         ........................................
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Exon_13_146         ........................................
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Exon_13_147            ........................................
Exon_13_148            ........................................
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B

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                  1620      1630      1640      1650      1660      1670
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Exon_14_2     GTGTCTGGTTGAGAAGGGAGATGTGGCCTTTGTGAAACACCAGACTGTCGAACAGAACACTGATG
Exon_14_3     ............................................................
Exon_14_4     ............................................................
Exon_14_5     ............................................................
Exon_14_6     ............................................................
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Exon_14_10    ............................................................
Exon_14_11    ............................................................
Exon_14_12    ...........................................R................
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Exon_14_14    ...........................................R................
Exon_14_15    ............................................................
Exon_14_16    ............................................................
Exon_14_17    ............................................................
Exon_14_18    ............................................................
Exon_14_19    ...........................................R................
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Exon_14_38    ............................................................
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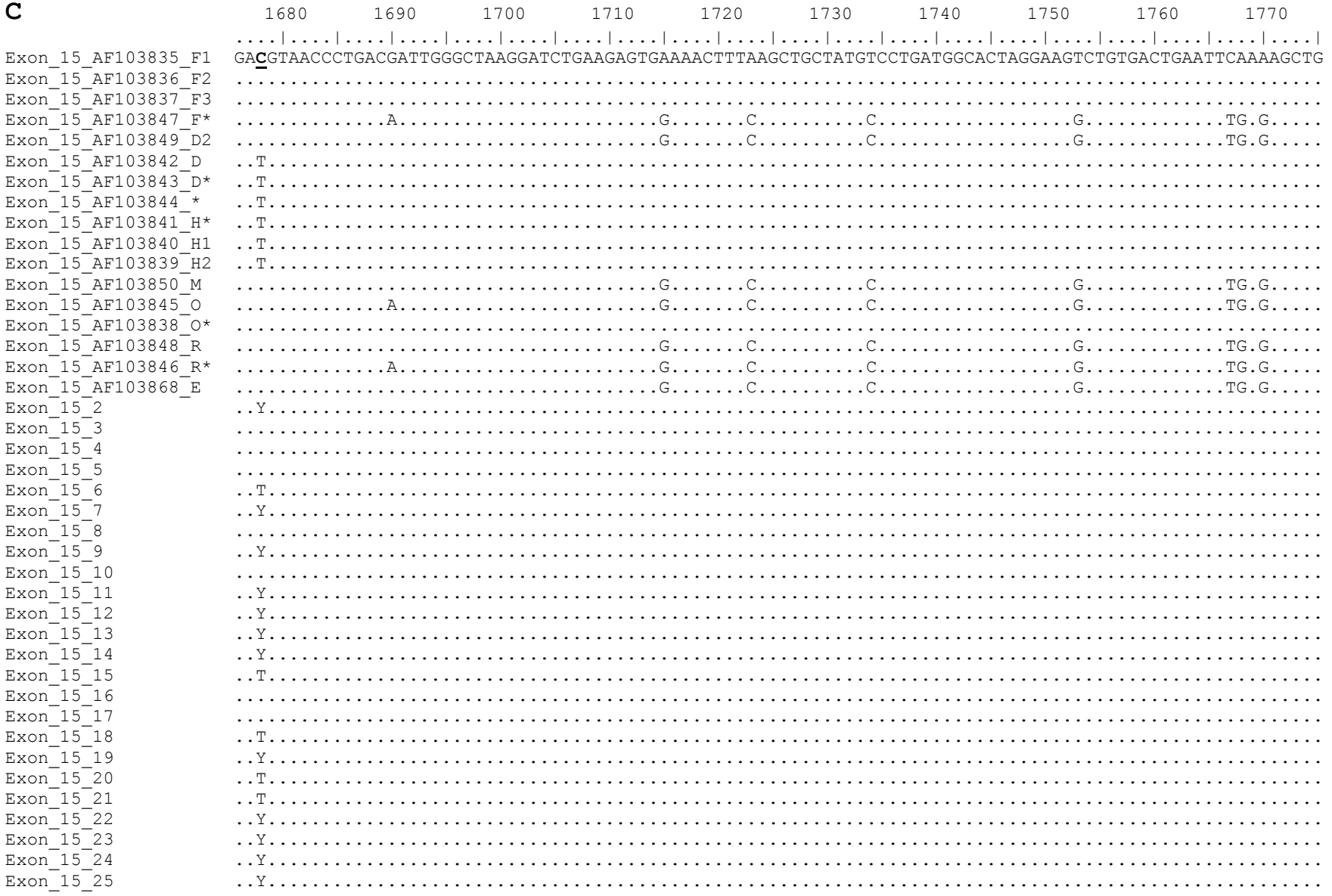
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Exon_15_AF103850_M
Exon_15_AF103845_O
Exon_15_AF103838_O*
Exon_15_AF103848_R
Exon_15_AF103846_R*
Exon_15_AF103868_E
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Exon_15_27          ..................................................................................................
Exon_15_28          ..Y...............................................................................................
Exon_15_29          ..Y...............................................................................................
Exon_15_30          ..T...............................................................................................
Exon_15_31          ..Y...............................................................................................
Exon_15_32          ..Y...............................................................................................
Exon_15_33          ..Y...............................................................................................
Exon_15_34          ..T...............................................................................................
Exon_15_35          ..T...............................................................................................
Exon_15_38          ..Y...............................................................................................
Exon_15_39          ..Y...............................................................................................
Exon_15_40          ..................................................................................................
Exon_15_41          ..................................................................................................
Exon_15_42          ..T...............................................................................................
Exon_15_43          ..................................................................................................
Exon_15_44          ..T...............................................................................................
Exon_15_45          ..T...............................................................................................
Exon_15_46          ..T...............................................................................................
Exon_15_47          ..Y...............................................................................................
Exon_15_48          ..T...............................................................................................
Exon_15_125         ..T...............................................................................................
Exon_15_126         ..................................................................................................
Exon_15_127         ..................................................................................................
Exon_15_128         ..T...............................................................................................
Exon_15_129         ..Y...............................................................................................
Exon_15_130         ..................................................................................................
Exon_15_131         ..................................................................................................
Exon_15_132         ..T...............................................................................................
Exon_15_133         ..Y...............................................................................................
Exon_15_134         ..................................................................................................
Exon_15_135         ..................................................................................................
Exon_15_136         ..T...............................................................................................
Exon_15_137         ..Y...............................................................................................
Exon_15_138         ..Y...............................................................................................
Exon_15_139         ..................................................................................................
Exon_15_140         ..Y...............................................................................................
Exon_15_141         ..Y...............................................................................................
Exon_15_142         ..Y...............................................................................................
Exon_15_143         ..................................................................................................
Exon_15_144         ..Y...............................................................................................
Exon_15_145         ..Y...............................................................................................
Exon_15_146         ..Y...............................................................................................
Exon_15_147         ..Y...............................................................................................
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Exon_15_148          ..................................................................................................
Exon_15_149          ..T...............................................................................................
Exon_15_150          ..T...............................................................................................
Exon_15_151          ..T...............................................................................................
Exon_15_152          ..................................................................................................
Exon_15_153          ..................................................................................................
Exon_15_154          ..T...............................................................................................
Exon_15_155          ..Y...............................................................................................

                        1780      1790      1800      1810      1820      1830      1840      1850      1860
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Exon_15_AF103835_F1   CTACCTAGCCCGAGCCCCGAATCATGCTGTAGTCTCACGGAAAGAAAAGGCAGCTTGTGTTTGCCAGGAGTTACACAACCAGCAG
Exon_15_AF103836_F2   ..................................................................................
Exon_15_AF103837_F3   ..................................................................................
Exon_15_AF103847_F*   .A........GA..............................................C..G.A.......G..........
Exon_15_AF103849_D2   .A........AA..............................................C..G.A.......G..........
Exon_15_AF103842_D    ..................................................................................
Exon_15_AF103843_D*   ..................................................................................
Exon_15_AF103844_*    ..................................................................................
Exon_15_AF103841_H*   ..................................................................................
Exon_15_AF103840_H1   ..................................................................................
Exon_15_AF103839_H2   ..................................................................................
Exon_15_AF103850_M    .A........AA..............................................C..G.A.......G..........
Exon_15_AF103845_O    .A........GA..............................................C..G.A.......G..........
Exon_15_AF103838_O*   ..................................................................................
Exon_15_AF103848_R    .A........AA..............................................C..G.A.......G..........
Exon_15_AF103846_R*   .A........GA..............................................C..G.A.......G..........
Exon_15_AF103868_E    .A........GA..............................................C..G.A......TG..........
Exon_15_2             ..................................................................................
Exon_15_3             ..................................................................................
Exon_15_4             ..................................................................................
Exon_15_5             ..................................................................................
Exon_15_6             ..................................................................................
Exon_15_7             ..................................................................................
Exon_15_8             ..................................................................................
Exon_15_9             ..................................................................................
Exon_15_10            ..................................................................................
Exon_15_11            ..................................................................................
Exon_15_12            ..................................................................................
Exon_15_13            ..................................................................................
Exon_15_14            ..................................................................................
Exon_15_15            ..................................................................................
Exon_15_16            ..................................................................................
Exon_15_17            ..................................................................................
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Exon_15_18 .....
Exon_15_19 .....
Exon_15_20 .....
Exon_15_21 .....
Exon_15_22 .....
Exon_15_23 .....
Exon_15_24 .....
Exon_15_25 .....
Exon_15_26 .....
Exon_15_27 .....
Exon_15_28 .....
Exon_15_29 .....
Exon_15_30 .....
Exon_15_31 .....
Exon_15_32 .....
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Exon_15_34 .....
Exon_15_35 .....
Exon_15_38 .....
Exon_15_39 .....
Exon_15_40 .....
Exon_15_41 .....
Exon_15_42 .....
Exon_15_43 .....
Exon_15_44 .....
Exon_15_45 .....
Exon_15_46 .....
Exon_15_47 .....
Exon_15_48 .....
Exon_15_125 .....
Exon_15_126 .....
Exon_15_127 .....
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Exon_15_129 .....
Exon_15_130 .....
Exon_15_131 .....
Exon_15_132 .....
Exon_15_133 .....
Exon_15_134 .....
Exon_15_135 .....
Exon_15_136 .....
Exon_15_137 .....
Exon_15_138 .....
Exon_15_139 .....

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Exon_15_140          ..........................................................................
Exon_15_141          ..........................................................................
Exon_15_142          ..........................................................................
Exon_15_143          ..........................................................................
Exon_15_144          ..........................................................................
Exon_15_145          ..........................................................................
Exon_15_146          ..........................................................................
Exon_15_147          ..........................................................................
Exon_15_148          ..........................................................................
Exon_15_149          ..........................................................................
Exon_15_150          ..........................................................................
Exon_15_151          ..........................................................................
Exon_15_152          ..........................................................................
Exon_15_153          ..........................................................................
Exon_15_154          ..........................................................................
Exon_15_155          ..........................................................................
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D

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                        1870      1880      1890      1900      1910      1920      1930      1940      1950      1960
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Exon_16_AF103851.2_F1 GCTTCGTATGGAAAAAATGGAAGTCACTGCCCAGACAAGTTTTGTTTGTTCCAGTCAGCCACCAAGGACCTTCTGTTCAGGGATGACACACAATGTTTGG
Exon_16_AF103852.2_F2 ..........................................................................................
Exon_16_AF185781_F3   ..........................................................................................
Exon_16_AF103863_F*   ..........................................................................................
Exon_16_AF103865_D2   ..........................................................T.......................................
Exon_16_AF103853.2_D  ..........................................................................................
Exon_16_AF103857_D*   ..........................................................................................
Exon_16_AF103855.2_*  ..........................................................................................
Exon_16_AF103856_H*   ..........................................................................................
Exon_16_AF103861_H1   ..........................................................................................
Exon_16_AF103854.2_H2 ..........................................................................................
Exon_16_AF103864_M    ..........................................................T.......................................
Exon_16_AF103859_O    ..........................................................................................
Exon_16_AF103862_O*   ..........................................................................................
Exon_16_AF103858_R    ..........................................................................................
Exon_16_AF103860_R*   ..........................................................................................
Exon_16_AF103866_E    ......................G...........................................................G.........
Exon_16_AF185782_G    ..........................................................................................
Exon_16_11            ..........................................................................................
Exon_16_12            ..........................................................................................
Exon_16_13            ..........................................................................................
Exon_16_14            ..........................................................................................
Exon_16_15            ..........................................................................................
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Exon_16_16      ................................................................................
Exon_16_17      ................................................................................
Exon_16_18      ................................................................................
Exon_16_19      ................................................................................
Exon_16_20      ................................................................................
Exon_16_21      ..............................................................Y.................
Exon_16_22      ................................................................................
Exon_16_23      ................................................................................
Exon_16_24      ................................................................................
Exon_16_25      ................................................................................
Exon_16_26      ................................................................................
Exon_16_27      ................................................................................
Exon_16_28      ................................................................................
Exon_16_29      ................................................................................
Exon_16_30      ................................................................................
Exon_16_31      ................................................................................
Exon_16_32      ................................................................................
Exon_16_33      ................................................................................
Exon_16_34      ................................................................................
Exon_16_35      ................................................................................
Exon_16_38      ................................................................................
Exon_16_39      ..............................................................R.................
Exon_16_40      ................................................................................
Exon_16_41      ..............................................................R.................
Exon_16_42      ................................................................................
Exon_16_43      ................................................................................
Exon_16_44      ..............................................................Y.................
Exon_16_45      ................................................................................
Exon_16_46      ................................................................................
Exon_16_47      ................................................................................
Exon_16_48      ................................................................................
Exon_16_138     ................................................................................
Exon_16_139     ................................................................................
Exon_16_140     ................................................................................
Exon_16_141     ................................................................................
Exon_16_142     ................................................................................
Exon_16_143     ................................................................................
Exon_16_144     ................................................................................
Exon_16_145     ................................................................................
Exon_16_146     ................................................................................
Exon_16_147     ................................................................................
Exon_16_148     ................................................................................
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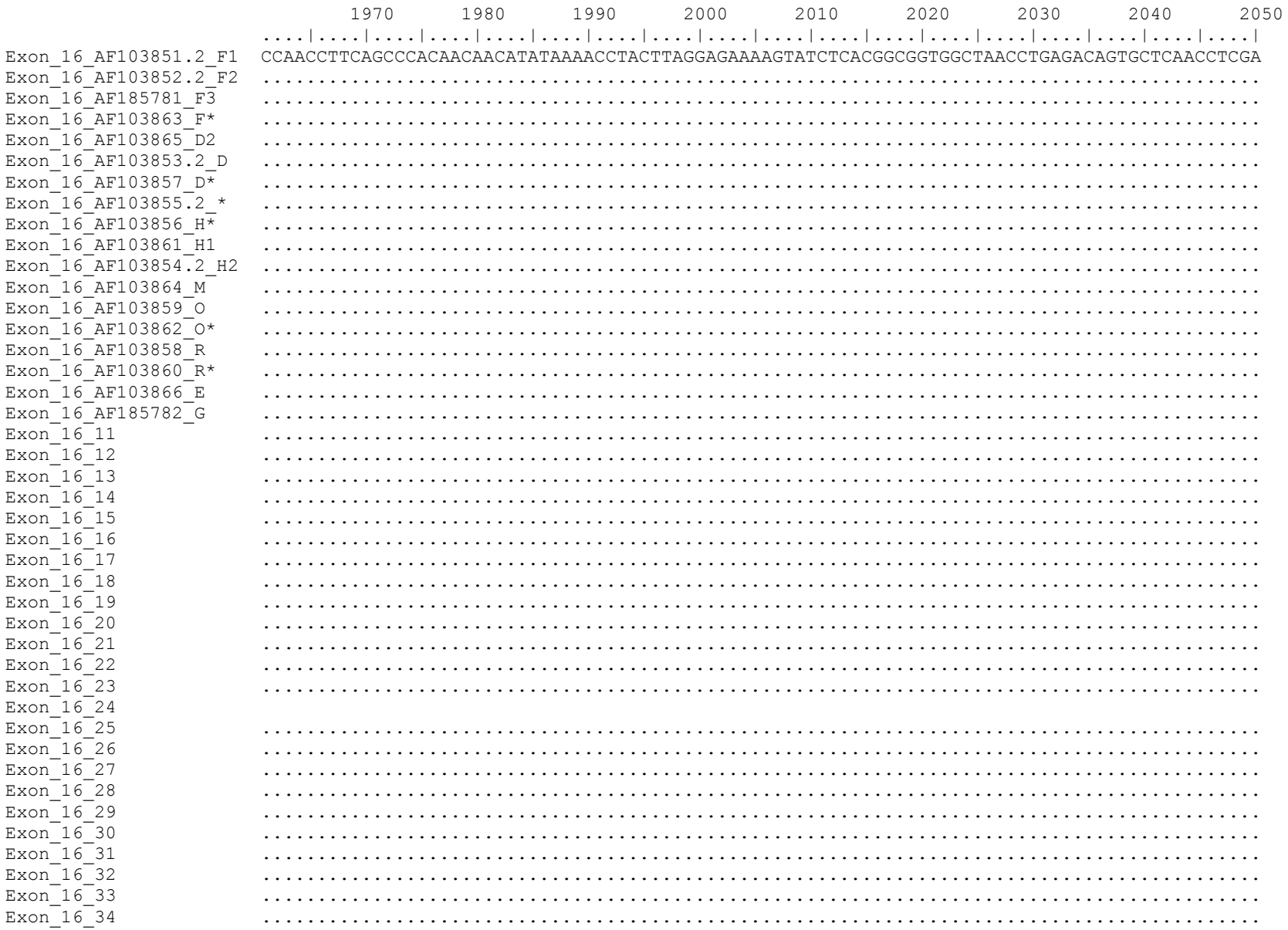
1970 1980 1990 2000 2010 2020 2030 2040 2050
Exon_16_AF103851.2_F1 CCAACCTTCAGCCCACAACAACATATAAAACCTACTTAGGAGAAAAGTATCTCACGGCGGTGGCTAACCTGAGACAGTGCTCAACCTCGA
Exon_16_AF103852.2_F2
Exon_16_AF185781_F3
Exon_16_AF103863_F*
Exon_16_AF103865_D2
Exon_16_AF103853.2_D
Exon_16_AF103857_D*
Exon_16_AF103855.2_*
Exon_16_AF103856_H*
Exon_16_AF103861_H1
Exon_16_AF103854.2_H2
Exon_16_AF103864_M
Exon_16_AF103859_O
Exon_16_AF103862_O*
Exon_16_AF103858_R
Exon_16_AF103860_R*
Exon_16_AF103866_E
Exon_16_AF185782_G
Exon_16_11
Exon_16_12
Exon_16_13
Exon_16_14
Exon_16_15
Exon_16_16
Exon_16_17
Exon_16_18
Exon_16_19
Exon_16_20
Exon_16_21
Exon_16_22
Exon_16_23
Exon_16_24
Exon_16_25
Exon_16_26
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Exon_16_29
Exon_16_30
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Exon_16_32
Exon_16_33
Exon_16_34

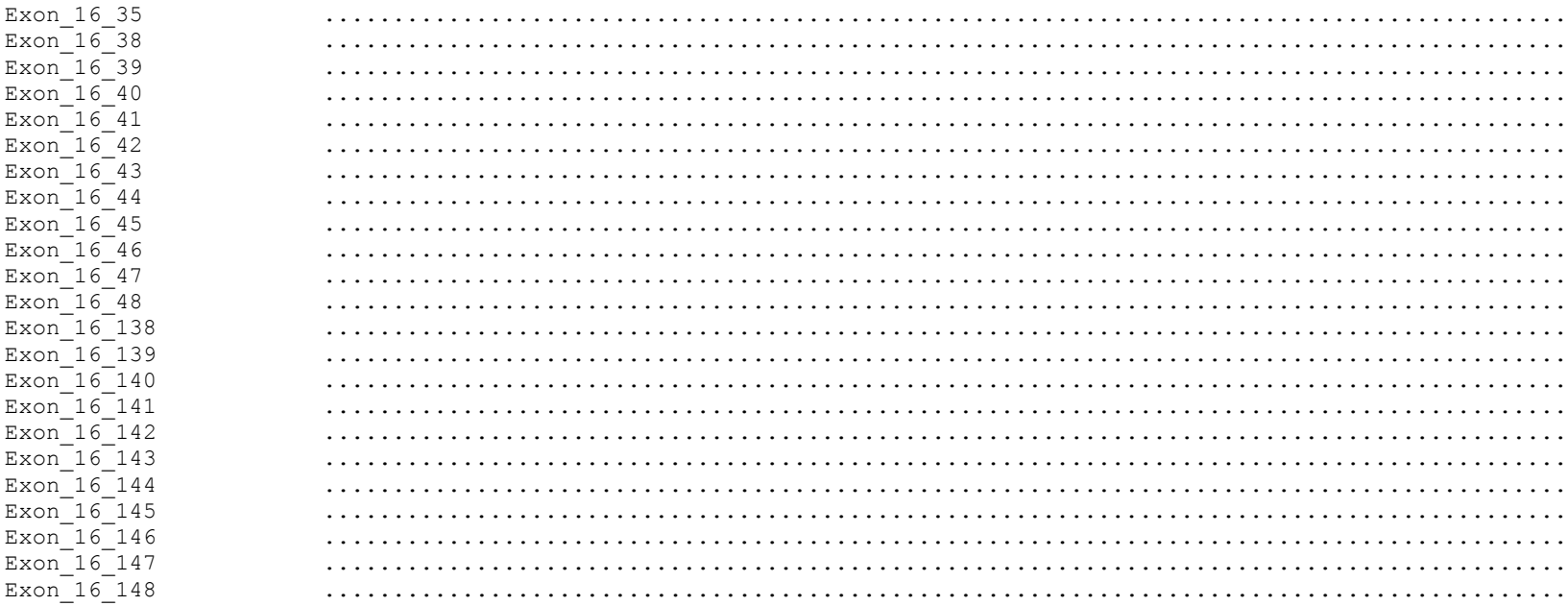

Figure 2. Nucleotide sequence alignment of the exons 13 (A), 14 (B), 15 (C), and 16 (D) (according to GenBank data base) of Tf gene. Reference sequences of Tf variants recorded in the GenBank for each exon were included for comparison. The Tf gene position is shown on the top.

3.2 Distribution of Tf alleles between breeds and phenotypic status

The SNPs observed here allowed us classify the animals in three variants: FF, DD, and FD. Once the SNPs found in the exons 13, 14 and 16 do not have discriminatory power among the known variants (**Figure 2**), the classification was made based on a single SNP found in the position 1678 (**C**GT/**T**GT) from exon 15 (14 for Ensemble). Therefore, sequences containing the codon **C**GT were classified as FF variant, those containing **T**GT were classified as DD, and the heterozygous sequences, which showed both options (**C/T**GT), were classified as FD variant (**Table 3**).

Table 3. Polymorphisms found in the sequences analyzed and respective allele classification.

ID	Breed	Status	Exon 13			Exon 14	Exon 15	Exon 16		Variant*
			1522	1542	1596	1660	1678	1914	1919	
2	BTB	Unaffected	NA	NA	NA	G	Y	NA	NA	FD
3	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
4	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
5	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
6	BTB	Unaffected	NA	NA	NA	G	T	NA	NA	DD
7	BTB	Affected	NA	NA	NA	G	Y	NA	NA	FD
8	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
9	BTB	Affected	NA	NA	NA	G	Y	NA	NA	FD
10	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
11	BSH	Unaffected	A	G	C	G	Y	G	C	FD
12	BSH	Affected	A	G	C	R	Y	G	C	FD
13	BSH	Unaffected	A	G	Y	G	Y	G	C	FD
14	BSH	Unaffected	A	G	C	R	Y	G	C	FD
15	BSH	Unaffected	A	G	C	G	T	G	C	D
16	BSH	Unaffected	A	G	C	G	C	G	C	FF
17	BSH	Unaffected	A	G	C	G	C	G	C	FF
18	BSH	Affected	A	G	C	G	T	G	C	DD
19	BSH	Unaffected	A	G	C	R	Y	G	C	FD
20	BSH	Unaffected	A	G	C	G	T	G	C	DD
21	BSH	Unaffected	A	G	C	A	T	G	Y	DD
22	BSH	Unaffected	A	G	C	G	Y	G	C	FD
23	BSH	Affected	A	G	C	R	Y	G	C	FD
24	BSH	Affected	A	G	C	G	Y	G	C	FD
25	BSH	Affected	A	G	C	G	Y	G	C	FD
26	BSH	Affected	A	G	C	G	C	G	C	FF
27	BSH	Affected	A	G	C	G	C	G	C	FF

28	BSH	Affected	A	G	C	R	Y	G	C	FD
29	BSH	Affected	G	G	C	R	Y	G	C	FD
30	BSH	Affected	A	G	C	R	T	G	C	DD
31	BSH	Affected	A	G	C	G	Y	G	C	FD
32	BSH	Affected	A	G	C	G	Y	G	C	FD
33	BSH	Affected	A	G	C	G	Y	G	C	FD
34	BSH	Affected	A	G	C	G	T	G	C	DD
35	BSH	Affected	A	G	C	A	T	G	C	DD
38	BSH	Unaffected	A	G	C	G	Y	G	C	FD
39	BSH	Unaffected	A	G	Y	G	Y	R	C	FD
40	BSH	Unaffected	A	G	C	G	C	G	C	FF
41	BSH	Unaffected	A	R	C	G	C	R	C	FF
42	BSH	Unaffected	A	G	C	G	T	G	C	DD
43	BSH	Unaffected	A	G	C	G	C	G	C	FF
44	BSH	Unaffected	A	G	C	G	T	G	Y	DD
45	BSH	Unaffected	A	G	C	A	T	G	C	DD
46	BSH	Unaffected	A	G	C	R	T	G	C	DD
47	BSH	Unaffected	A	G	C	G	Y	G	C	FD
48	BSH	Unaffected	A	G	C	G	T	G	C	DD
125	BTB	Affected	NA	NA	NA	G	T	NA	NA	DD
126	BTB	Affected	NA	NA	NA	G	C	NA	NA	FF
127	BTB	Affected	NA	NA	NA	G	C	NA	NA	FF
128	BTB	Affected	NA	NA	NA	G	T	NA	NA	DD
129	BTB	Affected	NA	NA	NA	G	Y	NA	NA	FD
130	BTB	Affected	NA	NA	NA	G	C	NA	NA	FF
131	BTB	Affected	NA	NA	NA	G	C	NA	NA	FF
132	BTB	Affected	NA	NA	NA	G	T	NA	NA	DD
133	BTB	Affected	NA	NA	NA	G	Y	NA	NA	FD
134	BTB	Affected	NA	NA	NA	G	C	NA	NA	FF
135	BTB	Affected	NA	NA	NA	G	C	NA	NA	FF
136	BTB	Affected	NA	NA	NA	G	T	NA	NA	DD
137	BTB	Affected	NA	NA	NA	G	Y	NA	NA	FD
138	BSH	Unaffected	A	G	C	R	Y	G	C	FD
139	BSH	Affected	A	R	C	G	C	G	C	FF
140	BSH	Unaffected	A	G	C	G	Y	G	C	FD
141	BSH	Unaffected	A	G	C	R	Y	G	C	FD
142	BSH	Affected	A	R	C	R	Y	G	C	FD
143	BSH	Unaffected	A	G	C	G	C	G	C	FF
144	BSH	Unaffected	A	G	C	G	Y	G	C	FD
145	BSH	Unaffected	A	G	C	R	Y	G	C	FD
146	BSH	Unaffected	A	R	C	G	Y	G	C	FD
147	BSH	Unaffected	A	G	C	G	Y	G	C	FD
148	BSH	Unaffected	A	G	C	G	C	G	C	FF
149	BTB	Unaffected	NA	NA	NA	G	T	NA	NA	DD
150	BTB	Unaffected	NA	NA	NA	G	T	NA	NA	DD
151	BTB	Unaffected	NA	NA	NA	G	T	NA	NA	DD

152	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
153	BTB	Unaffected	NA	NA	NA	G	C	NA	NA	FF
154	BTB	Unaffected	NA	NA	NA	G	T	NA	NA	DD
155	BTB	Unaffected	NA	NA	NA	G	Y	NA	NA	FD

* Alleles were determined based on exon 15 polymorphism.
NA: denotes not analyzed.

The frequencies of Tf alleles between BTB and BSH may be observed in the **Table 4**. FF variant seems predominating in BTB horses, while FD seems predominating in BSH. DD variant shows similarly distributed in both breeds (around 0.30 relative frequency).

Table 4. Tf alleles relative and absolute frequencies among breeds

Breed	**Alleles**			**Total**
	FF	DD	FD	
BTB	**0.45 (13)**	0.31 (9)	0.24 (7)	1 (29)
BSH	0.21 (10)	0.26 (12)	**0.53 (25)**	1 (47)
BTB + BSH	0.30 (23)	0.28 (21)	0.42 (33)	1 (76)

Considering the phenotypic status, we observed a predominance of FF variant in BTB affected (0.40 relative frequency) and unaffected (0.50 relative frequency) foals, and FD allele in BSH affected (0.59 relative frequency) and unaffected (0.50 relative frequency) foals (**Table 5**). Analyzing FF and FD together (FF + FD) we verified a high proportion of this variants in *R. equi* affected foals in both breeds (**Table 5**). The D allele seems to be more uncommon in the breeds evaluated.

Table 5. Tf alleles relative and absolute frequencies for each clinical phenotype.

Allele	**Unaffected**			**Affected**		
	BTB (n = 14)	BSH (n =30)	BTB + BSH (n =44)	BTB (n = 15)	BSH (n = 17)	BTB + BSH (n = 32)
FF	0.50 (7)	0.23 (7)	0.32 (14)	**0.40 (6)**	0.18 (3)	0.28 (9)
DD	0.36 (5)	0.27 (8)	0.30 (13)	0.27 (4)	0.24 (4)	0.25 (8)
FD	0.14 (2)	0.5 (15)	0.39 (17)	0.33 (5)	**0.59 (10)**	**0.47 (15)**
FF + FD	0.65 (9)	0.73 (22)	0.70 (31)	0.73 (11)	0.76 (13)	0.75 (24)

4. Discussion

This is the first study evaluating the presence of polymorphisms in the Tf gene in BSH horses, other studies have analyzed molecular typification of Tf gene in BTB horses (Brandon et al., 1999; Carpenter and Broad, 1994; Mousel et al., 2003). Moreover, the present study evaluated the major number of exons for which all transcript was sequenced, and included two different horse breeds.

Our results showed that DD variant is similarly distributed between affected and unaffected foals. Consequently, the hypothesis that D allele confers resistance or tolerance to rhodococcosis (Mousel et al., 2003; Newton et al., 2007) may be not confirmed herein. Differently, we verified a high frequency of FF and FD variants in unaffected foals. Although the presence of FF was associated to susceptibility to *R. equi* pneumonia (Mousel et al., 2003), the exposition to virulent *R. equi* isolates may have been different in affected and unaffected foals. Even all farms had endemic status for equine rhodococcosis, it is impossible to predict accurately the individual animal exposure to *R. equi*.

Conversely, a high relative frequency of FF in BTB (0.40) and FD (0.75) for both breeds was observed in affected foals (**Table 5**). The Tf variants show autosomal co-dominant inheritance (Carpenter and Broad, 1993b), consequently both F and D alleles are expressed in heterozygote animal. According to Newton et al. (2007), FD variant behaved more like F than D allele. We believe that FD genotype may have some influence in predisposition to *R. equi* infection in the animals evaluated, once the genetic susceptibility based on the accumulation of F allele seems more plausible than genetic resistance based on D allele.

Additionally, the participation of F allele as component of cumulative factors associated to *R. equi* infection is an interesting thought, especially because studies have suggested that *R. equi* foal pneumonia has a polygenic profile (McQueen et al., 2015). At the same way that D allele alone should be not enough to prevent *R. equi* infection, the presence of F allele may be

not a determinant to disease. Assessing the Tf polymorphisms in subclinical animals could allow a strongest influence of these alleles in the disease outcome.

It is difficult to establish the real impact of genetic polymorphisms in disease susceptibility. Throughout evolution of the species several mutations have culminated in a set of genotypic alteration with variable influence on the phenotype. The functional significance of the Tf polymorphisms needs to be discussed and the premise that F allele represents a variant less competitive for Fe remains to be determined. Studies addressing genetic susceptibility in horses have some advantage due to high pedigree completeness level and high rate of parentage control in many breeds. After statistical analysis we will be able to confirm or not the hypothesis that Tf alleles have influence on genetic susceptibility to *R. equi* pneumonia and perhaps improve our knowledge about *R. equi* ability to compete with Tf for iron within macrophages (Miranda-CasoLuengo et al., 2012).

References

Aulchenko, Y.S., Ripke, S., Isaacs, A., van Duijn, C.M., 2007. GenABEL: an R library for genome-wide association analysis. Bioinformatics 23, 1294-1296.

Barber, M.F., Elde, N.C., 2014. Nutritional immunity. Escape from bacterial iron piracy through rapid evolution of transferrin. Science 346, 1362-1366.

Bou Ghanem, E.N., Clark, S., Roggensack, S.E., McIver, S.R., Alcaide, P., Haydon, P.G., Leong, J.M., 2015. Extracellular Adenosine Protects against Streptococcus pneumoniae Lung Infection by Regulating Pulmonary Neutrophil Recruitment. PLoS pathogens 11, e1005126.

Brandon, R.B., Giffard, J.M., Bell, K., 1999. Single nucleotide polymorphisms in the equine transferrin gene. Animal genetics 30, 439-443.

Carpenter, M.A., Broad, T.E., 1993a. The cDNA sequence of horse transferrin. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1173, 230-232.

Carpenter, M.A., Broad, T.E., 1993b. The cDNA sequence of horse transferrin. Biochimica et biophysica acta 1173, 230-232.

Carpenter, M.A., Broad, T.E., 1994. Polymorphism in the coding sequence of the horse transferrin gene. Genome / National Research Council Canada = Genome / Conseil national de recherches Canada 37, 157-165.

Chaffin, M.K., Cohen, N.D., Martens, R.J., 2003. Evaluation of equine breeding farm characteristics as risk factors for development of Rhodococcus equi pneumonia in foals. Journal of the American Veterinary Medical Association 222, 467-475.

Cho, Y.J., Cunnick, J.M., Yi, S.J., Kaartinen, V., Groffen, J., Heisterkamp, N., 2007. Abr and Bcr, two homologous Rac GTPase-activating proteins, control multiple cellular functions of murine macrophages. Molecular and cellular biology 27, 899-911.

Cohen, N.D., Chaffin, M.K., Kuskie, K.R., Syndergaard, M.K., Blodgett, G.P., Takai, S., 2013. Association of perinatal exposure to airborne Rhodococcus equi with risk of pneumonia caused by R equi in foals. American journal of veterinary research 74, 102-109.

Cunnick, J.M., Schmidhuber, S., Chen, G., Yu, M., Yi, S.J., Cho, Y.J., Kaartinen, V., Minoo, P., Warburton, D., Groffen, J., Heisterkamp, N., 2009. Bcr and Abr cooperate in negatively regulating acute inflammatory responses. Molecular and cellular biology 29, 5742-5750.

Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic acids research 30, 207-210.

Esteso, G., Luzon, E., Sarmiento, E., Gomez-Caro, R., Steinle, A., Murphy, G., Carbone, J., Vales-Gomez, M., Reyburn, H.T., 2014. Altered microRNA expression after infection with human cytomegalovirus leads to TIMP3 downregulation and increased shedding of metalloprotease substrates, including MICA. Journal of immunology 193, 1344-1352.

Giguère, S., Cohen, N.D., Chaffin, M.K., Hines, S.A., Hondalus, M.K., Prescott, J.F., Slovis, N.M., 2011. Rhodococcus equi: clinical manifestations, virulence, and immunity. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 25, 1221-1230.

Go, Y.Y., Bailey, E., Cook, D.G., Coleman, S.J., Macleod, J.N., Chen, K.C., Timoney, P.J., Balasuriya, U.B., 2011. Genome-wide association study among four horse breeds identifies a common haplotype associated with in vitro CD3+ T cell susceptibility/resistance to equine arteritis virus infection. Journal of virology 85, 13174-13184.

Halbert, N.D., Cohen, N.D., Slovis, N.M., Faircloth, J., Martens, R.J., 2006. Variations in equid SLC11A1 (NRAMP1) genes and associations with Rhodococcus equi pneumonia in horses. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 20, 974-979.

Hines, M.T., Paasch, K.M., Alperin, D.C., Palmer, G.H., Westhoff, N.C., Hines, S.A., 2001. Immunity to Rhodococcus equi: antigen-specific recall responses in the lungs of adult horses. Veterinary immunology and immunopathology 79, 101-114.

Hondalus, M.K., Diamond, M.S., Rosenthal, L.A., Springer, T.A., Mosser, D.M., 1993. The intracellular bacterium Rhodococcus equi requires Mac-1 to bind to mammalian cells. Infection and immunity 61, 2919-2929.

Hondalus, M.K., Mosser, D.M., 1994. Survival and replication of Rhodococcus equi in macrophages. Infection and immunity 62, 4167-4175.

Horin, P., Osickova, J., Necesankova, M., Matiasovic, J., Musilova, P., Kubickova, S., Hubertova, D., Vyskocil, M., Rubes, J., 2008. Single nucleotide polymorphisms of interleukin-1 beta related genes and their associations with infection in the horse. Developments in biologicals 132, 347-351.

Horin, P., Sabakova, K., Futas, J., Vychodilova, L., Necesankova, M., 2010. Immunity-related gene single nucleotide polymorphisms associated with Rhodococcus equi infection in foals. International journal of immunogenetics 37, 67-71.

Horin, P., Smola, J., Matiasovic, J., Vyskocil, M., Lukeszova, L., Tomanova, K., Kralik, P., Glasnak, V., Schroffelova, D., Knoll, A., Sedlinska, M., Krenkova, L., Jahn, P., 2004. Polymorphisms in equine immune response genes and their associations with

infections. Mammalian genome : official journal of the International Mammalian Genome Society 15, 843-850.

Letek, M., Gonzalez, P., Macarthur, I., Rodriguez, H., Freeman, T.C., Valero-Rello, A., Blanco, M., Buckley, T., Cherevach, I., Fahey, R., Hapeshi, A., Holdstock, J., Leadon, D., Navas, J., Ocampo, A., Quail, M.A., Sanders, M., Scortti, M.M., Prescott, J.F., Fogarty, U., Meijer, W.G., Parkhill, J., Bentley, S.D., Vazquez-Boland, J.A., 2010. The genome of a pathogenic rhodococcus: cooptive virulence underpinned by key gene acquisitions. PLoS genetics 6, e1001145.

Liu, T.W., Ho, C.W., Huang, H.H., Chang, S.M., Popat, S.D., Wang, Y.T., Wu, M.S., Chen, Y.J., Lin, C.H., 2009. Role for alpha-L-fucosidase in the control of Helicobacter pylori-infected gastric cancer cells. Proceedings of the National Academy of Sciences of the United States of America 106, 14581-14586.

Martens, R.J., Cohen, N.D., Jones, S.L., Moore, T.A., Edwards, J.F., 2005. Protective role of neutrophils in mice experimentally infected with Rhodococcus equi. Infection and immunity 73, 7040-7042.

McGarvey, J.A., Wagner, D., Bermudez, L.E., 2004. Differential gene expression in mononuclear phagocytes infected with pathogenic and non-pathogenic mycobacteria. Clinical and experimental immunology 136, 490-500.

McQueen, C.M., Dindot, S.V., Foster, M.J., Cohen, N.D., 2015. Genetic Susceptibility to Rhodococcus equi. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 29, 1648-1659.

McQueen, C.M., Doan, R., Dindot, S.V., Bourquin, J.R., Zlatev, Z.Z., Chaffin, M.K., Blodgett, G.P., Ivanov, I., Cohen, N.D., 2014. Identification of genomic loci associated with Rhodococcus equi susceptibility in foals. PloS one 9, e98710.

Miranda-CasoLuengo, R., Coulson, G.B., Miranda-CasoLuengo, A., Vázquez-Boland, J.A., Hondalus, M.K., Meijer, W.G., 2012. The Hydroxamate Siderophore Rhequichelin Is Required for Virulence of the Pathogenic Actinomycete Rhodococcus equi. Infection and immunity 80, 4106-4114.

Mousel, M.R., Harrison, L., Donahue, J.M., Bailey, E., 2003. Rhodococcus equi and genetic susceptibility: assessing transferrin genotypes from paraffin-embedded tissues. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 15, 470-472.

Muscatello, G., Leadon, D.P., Klayt, M., Ocampo-Sosa, A., Lewis, D.A., Fogarty, U., Buckley, T., Gilkerson, J.R., Meijer, W.G., Vazquez-Boland, J.A., 2007. Rhodococcus equi infection in foals: the science of 'rattles'. Equine veterinary journal 39, 470-478.

Newton, J.R., Woodt, J.L., Chanter, N., 2007. Evidence for transferrin allele as a host-level risk factor in naturally occurring equine respiratory disease: a preliminary study. Equine veterinary journal 39, 164-171.

Paine, R., 3rd, Morris, S.B., Jin, H., Baleeiro, C.E., Wilcoxen, S.E., 2002. ICAM-1 facilitates alveolar macrophage phagocytic activity through effects on migration over the AEC surface. American journal of physiology. Lung cellular and molecular physiology 283, L180-187.

Prescott, J.F., 1991. Rhodococcus equi: an animal and human pathogen. Clinical microbiology reviews 4, 20-34.

Prescott, J.F., Meijer, W.G., Vázquez-Boland, J.A. 2010. Rhodococcus equi, In: Gyles, C.L., Prescott John F, Songer JG, Thoen, Charles O (Ed.) Pathogenesis of Bacterial Infections in Animals. Blackwell Publishing, Ames, 149-166.

Price, A.L., Zaitlen, N.A., Reich, D., Patterson, N., 2010. New approaches to population stratification in genome-wide association studies. Nature reviews. Genetics 11, 459-463.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J., Sham, P.C., 2007. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics 81, 559-575.

Raudsepp, T., McCue, M.E., Das, P.J., Dobson, L., Vishnoi, M., Fritz, K.L., Schaefer, R., Rendahl, A.K., Derr, J.N., Love, C.C., Varner, D.D., Chowdhary, B.P., 2012. Genome-wide association study implicates testis-sperm specific FKBP6 as a susceptibility locus for impaired acrosome reaction in stallions. PLoS genetics 8, e1003139.

Staden, R., 1996. The Staden sequence analysis package. Molecular biotechnology 5, 233-241.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular biology and evolution 24, 1596-1599.

Venner, M., Reinhold, B., Beyerbach, M., Feige, K., 2009. Efficacy of azithromycin in preventing pulmonary abscesses in foals. Veterinary journal 179, 301-303.

Wellcome Trust Case Control, C., 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447, 661-678.

Whitfield-Cargile, C.M., Cohen, N.D., Suchodolski, J., Chaffin, M.K., McQueen, C.M., Arnold, C.E., Dowd, S.E., Blodgett, G.P., 2015. Composition and Diversity of the Fecal Microbiome and Inferred Fecal Metagenome Does Not Predict Subsequent Pneumonia Caused by Rhodococcus equi in Foals. PloS one 10, e0136586.

Yamamoto, S., Shimizu, S., Kiyonaka, S., Takahashi, N., Wajima, T., Hara, Y., Negoro, T., Hiroi, T., Kiuchi, Y., Okada, T., Kaneko, S., Lange, I., Fleig, A., Penner, R., Nishi, M., Takeshima, H., Mori, Y., 2008. TRPM2-mediated Ca2+influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. Nature medicine 14, 738-747.