UNIVERSIDADE DE PASSO FUNDO
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOEXPERIMENTAÇÃO

NOVOS CONHECIMENTOS SOBRE AS PROPRIEDADES FUNCIONAIS E DE REATIVIDADE CRUZADA DE ANTICORPOS GERADOS CONTRA A PROTEÍNA TbpB DE *Haemophilus parasuis*

DISSERTAÇÃO DE MESTRADO

Bibiana Martins Barasuol

Passo Fundo, RS, Brasil
2016

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Bibiana Martins Barasuol

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioexperimentação, Área de Concentração em Bioexperimentação, da Faculdade de Agronomia e Medicina Veterinária da Universidade de Passo Fundo (UPF), como requisito parcial para a obtenção do grau de **Mestre em Bioexperimentação**

Orientador: Prof. Rafael Frandoloso

Passo Fundo, RS, Brasil
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Elaborada por
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Como requisito parcial para a obtenção do grau de
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“A sabedoria é essencial para que o desconhecido, o inexaurível, tome forma.”

Jiddu Krishnamurti

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3. CAPÍTULO 1

LISTA ABREVIATURAS

µg	Micrograma
µl	Microlitro
Ac	Anticorpo
App	*Actinobacillus pleuropneumoniae*
Ass	*Actinobacillus suis*
BCRs	Receptores de células B
BSA	Albumina sérica bovina
CC	Complemento de cobaio
CDTS	Toxinas de distinção citoletal
CFA	Adjuvante completo de Freund
CSAB	Tampão de ativação do sistema complemento
DG	Doença de Glässer
ELISA	Ensaio Imunoenzimático
FITC	Isotiocianato de fluoresceína
Hps	*Haemophilus parasuis*
IFA	Adjuvante incompleto de Freund
IgAs	Imunoglobulinas A
kDa	Quilo Dalton
mL	Mililitro
mM	Milimolar
NAD	Nicotinamida adenina dinucleótido
nm	Nanômetro
Omp	Proteína de membrana externa
PBMC	Células mononucleares de sangue periférico
PBS	Solução salina fosfatada
PBST	Solução salina fosfatada + Tween 20
PMN	Polimorfonucleares
pTf	Transferrina suína
RPM	Rotações por minuto
RPMI	Meio de cultivo Roswell Park Memorial Institute
SFB	Soro fetal bovino
Sh	Sistema hemolítico
SRBC	Anticorpos contra hemácia de ovelha
SV	Sorovar
TbpA	Proteína de união à transferrina A
TbpB	Proteína de união à transferrina B
TbpB-I	TbpB intacta (wild type)
TbpB-M	TbpB mutante
TbpB-C	Lóbulo C da TbpB
TbpB-Nm	Lóbulo N mutante da TbpB
TCRs	Receptores de células T
VCSC	Via clássica do sistema complemento

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo
Novos conhecimentos sobre as propriedades funcionais e de reatividade cruzada de anticorpos gerados contra a proteína TbpB de *Haemophilus parasuis*
Autor (a): Bibiana Martins Barasuol
Orientador: Rafael Frandoloso
Passo Fundo, 05 de agosto de 2016

Neste estudo, através de ensaios *in vitro*, apresentamos a caracterização funcional de anticorpos contra a proteína de união à transferrina B (TbpB) de *Haemophilus parasuis.* Quatro antígenos recombinantes baseados na estrutura da proteína TbpB foram desenvolvidos e avaliados neste estudo. As proteínas TbpB intacta (TbpB-I$^{20\text{-}528aa}$), TbpB mutante [TbpB-M$^{26\text{-}528aa}$ (Frandoloso, et al. 2015)] e os subdomínios (lóbulos) N (TbpB-Nm$^{20\text{-}282aa}$, versão mutante) e C (TbpB-C$^{283\text{-}528aa}$) da TbpB de *H. parasuis*, sorovar 5, cepa Nagasaki, foram clonadas no vetor de expressão pET20 e expressadas em células de *Escherichia coli* cepa ER2566. A purificação dos antígenos foi realizada mediante cromatografia líquida de proteína. Suínos foram imunizados com os antígenos recombinantes potencializados com o adjuvante Montanide IMS 2215 VG PR e, a cinética da resposta de anticorpos foi analisada através de um ELISA indireto. A capacidade dos antissoros de reconhecerem 5 cepas virulentas de *H. parasuis* (Nº4 – Sorovar (SV) 1; SW124 – SV4; Nagasaki – SV5; H425 – SV12; 84-22113 – SV14 e 84-15995 – SV15) foi avaliada por citometria de fluxo, como também o efeito opsonizante dos anticorpos durante a fagocitose de *H. parasuis* realizada por neutrófilos suínos. Por último, analisou-se a capacidade dos diferentes antissoros de fixar a via clássica do sistema do complemento e, de produzir a morte das bactérias através de um ensaio bactericida mediado por anticorpos. Os resultados apresentados neste estudo demonstram que todos os antígenos recombinantes foram imunogênicos em suínos e, que os títulos de anticorpos mais elevados foram induzidos pelos antígenos TbpB-M e TbpB-C. Com exceção do SV4, todos os demais sorovares (SV1, SV5, SV12, SV14 e SV15) foram altamente reconhecidos (60 – 90%) por um ou mais antissoros específicos, demonstrando que a proteína TbpB dos 5 sorovares virulentos analisados compartilham epítopos conservados tanto no lóbulo N e no lóbulo C da proteína TbpB. De maneira geral, o número de anticorpos associados às cepas avaliadas foi menor quando utilizou-se os antissoros produzidos com o antígeno TbpB-I. Interessantemente, observamos que a expressão homogênea da proteína TbpB nativa de *H. parasuis* (independente do sorovar) somente alcançada se o microrganismo for cultivado em condições restritivas de ferro. Com relação a fagocitose, observamos que o processo de opsonização, independente do tipo de anticorpo específico aumentou significativamente o número de neutrófilos com bactérias associadas, bem como o número de bactérias capturadas por cada neutrófilos. A porcentagem de neutrófilos com bactérias internalizadas foi significativamente maior quando os microrganismos estavam opsonizados, no entanto, este processo parece ser mais lento contra o SV12. Surpreendentemente, nenhum dos antissoros suínos foi capaz de ativar a via clássica do sistema do complemento (VCSC), sugerindo que o adjuvante Montanide IMS 2215 VG induza um isotipo de anticorpo com reduzida capacidade funcional em suínos. De maneira contrária, anticorpos produzidos em camundongos contra as proteínas TbpB-M TbpB-Nm e TbpB-C potencializadas com os adjuvantes de Freund e ou Montanide Gel 01 foram capazes de fixar VCSC contra todos os sorovares analisados. Em síntese, demonstramos neste estudo, a capacidade funcional dos anticorpos gerados contra 4 antígenos recombinantes baseados na

estrutura da proteína TbpB de *H. parasuis*. Os resultados apresentados são úteis para predizer *in vitro* o potencial de proteção homólogo e heterólogo de vacinas baseadas nestes antígenos.

Palavras – chave: Doença de Glässer; *Haemophilus parasuis*; TbpB; Fagocitose; Sistema Complemento.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo
New insights about functional and cross-reactivity properties of antibodies generated against recombinant TbpBs of *Haemophilus parasuis*
Author: Bibiana Martins Barasuol
Advisor: Rafael Frandoloso
Passo Fundo, august 5th, 2016

In this study, through in vitro assays, we present the functional characterization the antibody against transferrin binding protein B (TbpB) from *Haemophilus parasuis*. Four recombinant antigen-based on the protein structure of TbpB were developed and evaluated in this study. The intact TbpB protein (TbpB-I$^{20\text{-}528aa}$, mutant TbpB TbpB-M$^{26\text{-}528aa}$ and subdomains (lobes) N TbpB-Nm$^{20\text{-}282aa}$ mutant version) and C TbpB-C$^{283\text{-}528aa}$ of TbpB from *H. parasuis*, serotype 5, Nagasaki strain, were cloned into pET20 expression vector and expressed in cells of *Escherichia coli*, strain ER 2566. The recombinant antigens were purified by liquid chromatography. Pigs were immunized with recombinant antigens adjuvanted with the adjuvant Montanide IMS 2215 VG PR and, the kinetics of antibody response was assessed by indirect ELISA. The antisera capacity to recognize 6 virulent strains of *H. parasuis* (Nº4 – Serovar (SV) 1, SW124 – SV4, Nagasaki – SV5, H425 – SV12, 84-22113 – SV14 e 84-15995 – SV15) was analyzed by flow cytometry, as well as the effect of antibodies opsonization during the *H. parasuis* phagocytosis performed by swine neutrophils. Finally, we analyze the ability of different antisera to activate the classical pathway of the complement system and to produce the killing of bacteria through antibody-mediated bactericidal assay. The results presented in this study demonstrate that all recombinant antigens were immunogenic in pigs and, that higher titres of antibodies were induced by antigens TbpB-M and TbpB-C, respectively. Except for SV4, all others serovars (SV1, SV5, SV12, SV14 e SV15) were strongly recognized (60-90%) by one or more specific antisera, demonstrating that TbpB protein from 6 virulent serovars analyzed share conserved epitopes in both lobes, N and C of the TbpB protein. In general, the number of antibodies associated with the strains was lower when we used the antisera produced with TbpB-I antigen. Interestingly, we observed that the homogenous expression of native TbpB protein from *H. parasuis* (independently of serovar) is only achieved if the microorganism is growth in iron starvation. Regarding the phagocytosis, we observed that the opsonization process, independently of the specific type of antibody, have increased the number of neutrophils with associated bacteria, as well, the number of bacteria captured by each neutrophil. The percentage of neutrophils with internalized bacteria was significantly higher when the microorganism was opsonized, however, this process seems to be slower against the SV12. Surprisingly, none of the swine antisera was capable of activating the classical pathway of the complement system (CPCS), suggesting that the Montanide IMS 2215 VG adjuvant has induced an antibody isotype with reduced functional activity in pigs. On the other hand, antibodies produced in mice against the TbpB-M, TbpB-Nm and TbpB-C protein adjuvanted with Freund adjuvant and/or Montanide Gel 01 were able to activate CPCS against all sevorars included. In summary, we demonstrate in this study, the functional activity of specific antibodies generated against four recombinant antigens based on the structure of TbpB protein from *H. parasuis*. The results presented are useful to predict *in vitro* the capacity of homologous and heterologous protection of vaccines based on these antigens.

Keywords: Glässer Disease, *Haemophilus parasuis*, TbpB, Antibodies, Phagocytosis, Complement Systems.

1. INTRODUÇÃO

Haemophilus parasuis é uma bactéria Gram negativa, hospedeiro específico, comumente encontrada no trato respiratório superior de suínos convencionais. Este microrganismo é o agente etiológico da Doença de Glässer (DG), uma patologia inflamatória sistêmica que cursa com poliserosites, artrites e meningites (1). Infecções produzidas pelo *H. parasuis* tem emergido notavelmente nos últimos anos, e as perdas econômicas geradas por consequência da DG ressaltam a importância deste patógeno nos atuais sistemas intensivos de produção de suínos (2) .

Em razão da alta diversidade antigênica superficial do *H. parasuis*, vacinas formuladas com cepas inativadas possuem limitada capacidade de proteção heteróloga (3,4) representando um dos principais gargalos preventivos do setor. Como consequência, intensivas práticas de manejo são adotadas para prevenir o surgimento da DG, demandando do uso cada vez mais extensivos de antimicrobianos.

H. parasuis (*Hps*), *A. pleuropneumoniae* (*App*) e *A. suis* (Ass) são microrganismos que contém um especializado sistema de captação de ferro, o qual capta este elemento da transferrina do hospedeiro (5) e tem se mostrado crítico para a sobrevivência e produção de doença em suínos (6).

As proteínas que compõem o sistema de captação de ferro de *H. parasuis* são denominadas de Proteínas de União à Transferrina A (TbpA) e B (TbpB) (7). A TbpA é uma proteína integral de membrana externa intimamente relacionada com outros receptores TonB-dependentes que estão envolvidos com a aquisição de sideróforos e vitamina B12 (8). Sua função consiste em transportar íons de ferro através da membrana externa até o espaço periplasmático (9). A segunda proteína que compõe o receptor é a TbpB, uma lipoproteína exposta na superfície da membrana externa capaz de unir-se independentemente à transferrina (5,10).

Em razão da vital função deste receptor, fundamentalmente, por superar a nutrição imunológica do hospedeiro (11), as proteínas TbpA e B se converteram em alvos potenciais para o desenvolvimento de vacinas contra patógenos de interesse veterinário (12–14) e humano (9,15,16).

Recentemente, nós (17) desenvolvemos e caracterizamos estruturalmente a proteína TbpB de *Hps*, *App* e *Ass*, e demonstra seu potencial imunogênico e protetor em suínos desafiados experimentalmente com uma dose letal de *H. parasuis* SV5, cepa Nagasaki (14). Neste trabalho, caracterizamos os mecanismos funcionais mediados por um pianel de anticorpos específicos gerados contra quatro antígenos recombinantes baseados na estrutura da proteína TbpB da cepa Nagasaki de *H. parasuis*.

Os resultados obtidos estão descritos no capítulo um o qual se constitui em um artigo científico intitulado "Caracterização da resposta imune funcional induzida por antígenos recombinantes derivados da proteína TbpB de *Haemophilus parasuis*" e foi submetido para publicação no periódico *Vaccine*.

2. REVISÃO BIBLIOGRÁFICA

2.1 *Haemophilus parasuis*

Haemophilus parasuis é uma bactéria Gram negativa dependente de nicotinamida adenina dinucleótido (NAD) e integrante da família *Pasteurellaceae*. Morfologicamente, apresenta-se como pequenos bacilos, não hemolíticos, imóveis e com um metabolismo bioquímico que permite sua diferenciação de outras bactérias que infectam o trato respiratórios dos suínos (18).

H. parasuis coloniza o trato respiratório superior de leitões, através do contato direto com a fêmea logo após o nascimento. É encontrado exclusivamente em suínos, e normalmente causa infecção no trato respiratório superior, porém, em determinadas circunstâncias, algumas cepas virulentas migram para os pulmões causando pneumonia, ou, alcançam a corrente sanguínea causando um quadro inflamatório sistêmico, com presença de poliserosites e artrite, lesões características da Doença de Glässer (19). Sinais clínicos como febre, depressão, anorexia, articulações inchadas, dispnéia, e sinais neurológicos podem apresentar-se durante a infecção (3).

Fenotipicamente este microrganismo por ser classificado em 15 sorovares agrupados de acordo com a virulência: os sorovares 1, 5, 10, 12, 13 e 14 são classificados como altamente virulentos; os sorovares 2, 4 e 15 moderadamente virulentos; e os sorovares 3, 6, 7, 8, 9 e 11 classificados como não ou pouco virulentos (21).

2.1.1 Patogênese do *H. parasuis*

Em relação a patogênese, *H. parasuis* possui mecanismos para captar NAD a partir das células do trato respiratório e evadir as defesas de mucosa dos suínos. Com relação ao segundo, algumas cepas de *H. parasuis* sintetizam proteases que clivam especificamente as IgAs de mucosa, sugerindo que este seja um mecanismo crucial para as cepas virulentas possam alcançar a corrente circulatória e produzir doenças sistêmicas (22,23).

Após superar as defesas específicas de mucosa, a aderência aos tecidos do hospedeiro consiste em um passo vital para colonização e sobrevivência bacteriana. Estruturas presentes na superfície das bactérias denominadas de adesinas, interagem com receptores específicos presente nas células do hospedeiro e medeiam o processo. Neste contexto, *H. parasuis* possui algumas moléculas, como a VtaA [proteína da família "trimeric autotransporters (AT-2)], D15, Omp2, OmpA, OmpP5 e PalA, as quais promovem aderência do patógeno às células epiteliais e são alvos potenciais para o desenvolvimento de vacinas (24–26).

O *H. parasuis* também pode superar a resposta imune inata pulmonar, constituindo um passo fundamental na patogênese. Em mamíferos, a resposta imune inata do pulmão é a mais importante linha de defesa contra a invasão de patógenos respiratórios, sendo esta resposta, realizada através dos macrófagos alveolares, os quais realizam a fagocitose bacteriana e orquestram a resposta pró-inflamatória através da liberação de citocinas e quimioquinas pró-inflamatórias (27–31).

Neste cenário, o desenvolvimento de doença pulmonar por patógenos bacterianos fica condicionado a presença de fatores de virulência e mecanismos estratégicos de evasão da fagocitose e, neste particular, *H. parasuis* expressa duas proteínas nomeadas de VtaA8 e VtaA9 capazes de retardar este processo (32,33).

Em nível sistêmico, as toxinas de distinção citoletal (CDTS) produzidas pelas cepas virulentas de *H. parasuis* parecem atuar sobre as vias primitivas do sistema do complemento, conferindo-lhe resistência ao soro (25,32). Ainda, mediante mecanismos não conhecidos, este patógeno induz a depleção específica dos linfócitos TCRγδ periféricos (34), os quais constituem a subpopulação linfocitária mais numerosa presente no sangue periféricos de suínos jovens (35).

2.2 MECANISMO DE AQUISIÇÃO DE FERRO

H. parasuis e demais microrganismos das famílias *Pasteurellaceae* e *Neisseriaceae*, necessitam superar os mecanismos inatos da nutrição imunológica (11) para sobreviverem dentro dos seus hospedeiros.

Nos vertebrados o transporte de ferro é mediado pela transferrina, uma proteína que capta o ferro circulante e realiza a entrega deste elemento às células do organismo, através de um processo de endocitose mediada por receptores específicos. Ademais do seu papel principal, a transferrina contribui para a nutrição imunológica do hospedeiro, restringindo o ferro livre aos patógenos bacterianos (11).

Como consequência da restrição de ferro livre presente nos fluídos dos suínos, *H. parasuis*, *A. pleuropneumoniae* e *A. suis* desenvolveram um importante mecanismo de aquisição de ferro a partir da transferrina suína (pTf) (13).

Este mecanismo baseia-se num complexo receptor formado por duas proteínas localizadas na membrana externa do patógeno. A proteína de união à transferrina A (TbpA), consiste em uma proteína integral de membrana de aproximadamente 100 kDa e dependente energeticamente do complexo TonB. Esta proteína forma um poro transmembrana que conecta o espaço extracelular com o periplasmático (Fig.1-A). A proteína de união à transferrina B (TbpB), tem menor tamanho (60 - 80 kDa) e está ancorada na membrana externa por uma sequência de 18 aminoácidos hidrofóbicos (Fig. 1-B). A TbpB é responsável por captar a transferrina e transportá-la até a superfície da TbpA, a qual extrai o ferro da transferrina e transporta este elemento para o espaço periplsmático (Fig. 1-C) (9,15,36).

Estruturalmente, a TbpB está constituída por dois lóbulos denominados de N e C arquitetonicamente muito parecidos (Fig.1-B). Cada lóbulo apresenta-se na forma de barril formado por 8 lâminas betas flanqueadas por um domínio acessório ("handle") contendo quatro (N-lóbulo) e oito (C-lóbulo) lâminas betas, respectivamente (Fig. 2) (15).

Apesar da similaridade estrutural, os laços que emergem dos domínios acessórios bem como dos barris, conferem cargas eletrostáticas completamente diferentes aos lóbulos. Funcionalmente, o lóbulo N (altamente eletropositivo) tem alta afinidade pela transferrina suína (pTf), já o lóbulo C (eletronegativo) apenas consegue fixá-la (debilmente) (16).

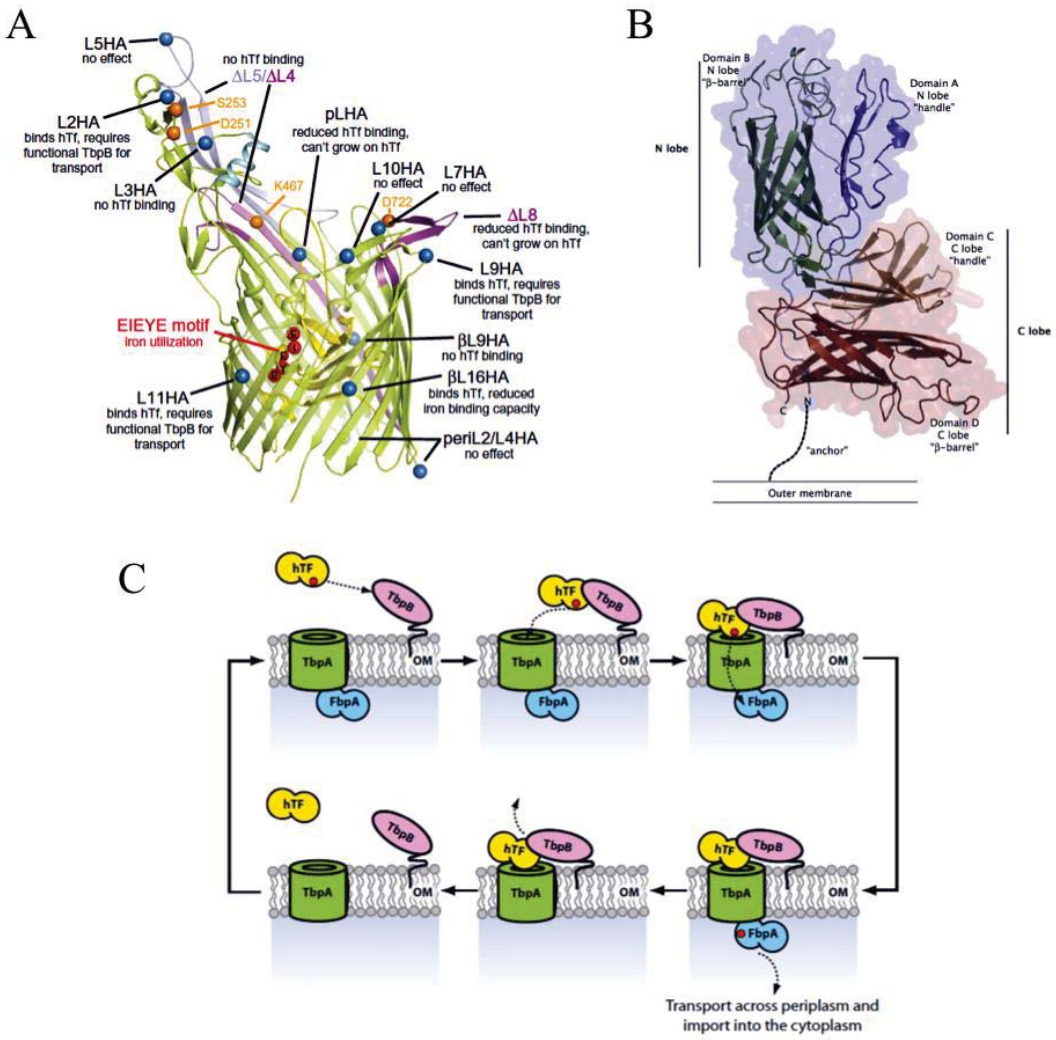

Figura 1. Sistema de captação de ferro de *H. parasuis*. A) Estrutura cristalográfica da proteína TbpA de *Neisseria meningitidis* (16). B) Estrutura cristalogrática da proteína TbpB de *Actinobacillus pleuropneumoniae* (15). C) Mecanismo passo a passo de aquisição de ferro de *N. Meningitidis* a partir da transferrina humana: primeiramente a TbpB (rosa) capta a transferrina humana (hTf) carregada com ferro (hTf representada em amarelo e o ferro como uma esfera vermelha) e a transporta para a superfície da TbpA (verde). A união com a transferrina induz uma mudança conformacional no domínio de encaixe (plug) da TbpA, formando um lugar transitório de união ao ferro. Posteriormente, o ferro é conduzido através das lâminas betas até o periplasma. No periplasma, a proteína de união ao ferro A (FbpA, cinza) capta o ferro e o transporta até a membrana citoplasmática. Depois que o ferro foi extraído da transferrina, a TbpB remove a apo-transferrina da superfície da TbpA, adota sua conformação original, e permite que um novo ciclo de captação de ferro ocorra (16).

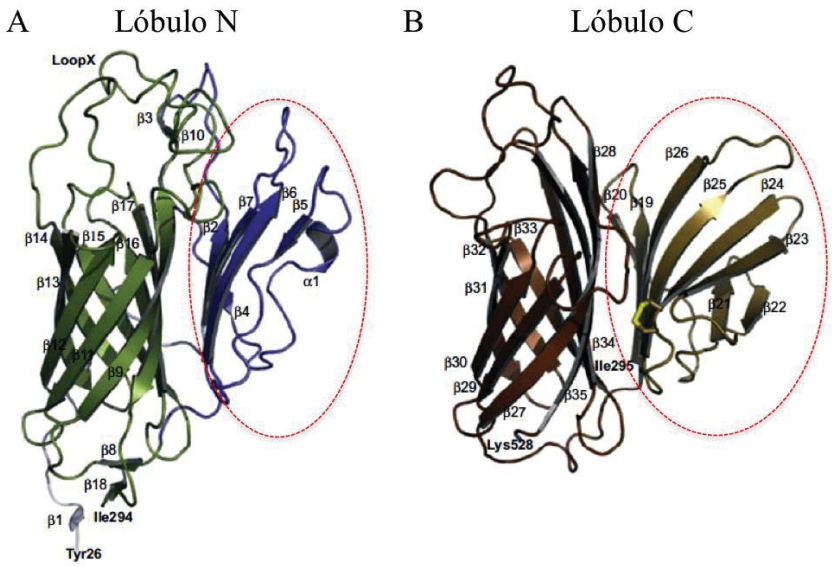

Figura 2. Estrutura cristalográfica dos lóbulos N e C da TbpB. A) representação da estrutura do lóbulo N incluindo os resíduos 47-284. B) representação da estrutura do lóbulo C incluindo os resíduos 290-526. Os domínios acessórios dos lóbulos N e C estão circulados em vermelho. Destaca-se que a diferença de tamanho dos dois lóbulos é em decorrência do número de lâminas betas dos domínios "handle" (15).

Recentemente, o complexo pTf–TbpB foi determinado, revelando que o processo de ligação da TbpB à transferrina não produz mudanças significativas na conformação do lóbulo N da TbpB ou no lóbulo C da transferrina suína (9,36). Por outro lado, a ligação da pTf à TbpA produz alterações significativas na conformação do lóbulo C da TbpB, resultando em uma separação substancial dos domínios C1 e C2 (9).

Com relação a conservação estrutural da proteína TbpB, destacam-se as regiões localizadas entre os domínios acessórios e as lâminas betas que foram o barril de cada um dos lóbulos, e os laços que flanqueiam a região inter lobular (15). Já em relação aos lóbulos, o lóbulo C apresenta-se de forma geral, bem mais conservado que o lóbulo N nos microrganismos *H. parasuis*, *A. pleuropneumoniae* e *A. suis* (37).

2.3 VACINAS BASEADAS NAS PROTEÍNAS TbpA E TbpB

As proteínas de união à transferrina são considerados alvos potenciais para o desenvolvimento de vacinas contra patógenos que adquirem o ferro a partir da transferrina do hospedeiro (14,38,39).

Neisseria meningitidis sorogrupo B (MenB) é uma bactéria extremamente patogênica e de alta diversidade genética (40). Enquanto estão disponíveis vacinas para proteger contra os serogrupos A, C, W135 e Y, não existe atualmente uma vacina comercial para conferir proteção contra a MenB, fundamentalmente pela composição da cápsula deste sorogrupo. Uma estratégia para o desenvolvimento de uma vacina contra a MenB é a utilização das proteínas que formam o sistema de captação de ferro deste microrganismo, considerado bem conservados e com grande potencial de promover ampla proteção contra as infecções meningocócicas (16).

Diversos estudos demonstram que as proteínas TbpA e TbpB são imprescindíveis para a sobrevivência de algumas bactérias Gram negativas de interesse humano e veterinário. Baltes, et al. (2002) (41), depois de delecionar os genes *tbpA* e *tbpB* do sorotipo 7 de *A. pleuropneumoniae*, comprovaram mediante um experimento de infecção por aerossóis, que a cepa mutante havia se convertido em avirulenta e incapaz de colonizar o trato respiratório dos suínos. Cornelissen, et al. (1992) (42). demonstraram que cepas mutante de *N. gonorrhoeae* deficientes do gene *tbpA* eram incapazes de crescer em presença de transferrina como única fonte de ferro. Resultados similares foram encontrados em *H. influenzae* (5) e *M. catarrhalis* (43).

O efeito imuno protetor da TbpB também foi demonstrado por Rossi-Campos, et al. (1992) (39), em um estudo de infecção com *A. pleuropneumoniae*. Potter, et al. (1999) avaliaram em terneiros diferentes formulações vacinais compostas pelas Tbps e mostraram que a associação dos antígenos TbpA e TbpB induziam altos títulos de anticorpos e conferiam completa proteção frente ao desafio experimental com *Mannheimia haemolytica* (44).

Nosso grupo, demonstrou que as proteínas TbpA e TbpB são alvos ideias para formulação de vacinas para a prevenção das infecções produzidas pelo *H. parasuis* (12–14). Estes antígenos, estimulam em suínos uma forte resposta de linfócitos B (13,14,34,45) com produção de anticorpos sistêmicos e de mucosa capazes de conferir aos animais imunizados o *status* de não portador do microrganismo após a infecção experimental(46)

Este histórico demonstra o potencial destes antígenos para compor a unidade antigênica de uma futura vacinal comercial desenhada para a prevenção de doenças produzidas por importantes patógenos veterinários e humanos. Neste trabalho, abordaremos a análise funcional da resposta imune humoral específica de suínos vacinados com 4 antígenos recombinantes derivados da proteína TbpB de *H. parasuis*, contribuindo para o entendimento do processo de defesa do hospedeiro contra este patógeno, e para a seleção de antígenos recombinantes com melhor potencial funcional.

3. CAPITULO 1

New insights about functional and cross-reactivity properties of antibodies generated against recombinant TbpBs of *Haemophilus parasuis*

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Abstract

Haemophilus parasuis is a bacteria well adapted to the upper respiratory tract of swine. During replication in the host the bacteria uses the transferrin binding protein B (TbpB) to steal iron molecules from pig's transferrin. Because iron is essential to bacterial replication, the TbpB of *H. parasuis* is highly conserved amongst its serovars, which makes it a suitable antigen candidate for vaccine development. In this study we analyzed the effector mechanisms of swine antibodies specific to four recombinant TbpB-based antigens: TbpB wild type (TbpB-I); TbpB mutant (TbpB-M), TbpB N lobule mutant (TbpB-Nm); and TbpB C lobule (TbpB-C). Groups of pigs were immunized with all antigens combined with Montanide IMS 2215 VG PR adjuvant and the antisera tested against reference strains 4 (SV1), SW124 (SV4), Nagasaki (SV5), H425 (SV12), 84-22113 (SV14) and 84-15995 (SV15). All *H. parasuis* serovars (except SV4) were highly (60 – 90%) recognized by one or more antisera. And, in general, the number of antibody molecules associated with these serovars was lowers when anti-TbpB-I antisera were used. Neutrophil-associated bacteria and the efficiency of phagocytosis were higher in the presence of antisera. Surprisingly, none of the swine antisera was able to activate the classical complement pathway (CCP). Antisera from mice immunized with TbpB-M or TbpB-C adjuvanted with Montanide IMS 2215 VGPR also failed to activate the CCP. In contrast, antisera from mice immunized with TbpB-M, TbpB-C or TbpB-Nm adjuvanted with Freund' complete adjuvant or Montanide Gel 01, were able to activate the CCP and kill *H. parasuis*. Our results indicated the potential of using different forms of recombinant TbpB as universal vaccine to induce functional protecting antibodies with cross-reactivity against heterologous strains of *H. parasuis*. Furthermore, we demonstrated that the induction

of antibody with important protecting properties such as the activation of the CCP is dependent on the type of adjuvant used.

Keywords: *Haemophilus parasuis*, TbpB; Vaccine, Antigenicity; Phagocytosis; Neutrophils, Complement System.

1. Introduction

Haemophilus parasuis is a Gram negative, host-specific bacteria commonly found in the upper respiratory tract of conventional raised pigs. This bacterium is the etiological agent of Glässer Disease (GD), a systemic inflammatory condition characterized by polyserositis, arthritis and meningitis [1]. Worldwide outbreaks of GD are on the raise and the economic losses associated with it are of major concern to swine producers and researchers [2].

There are at least 15 antigenically distinct serovars of *H. parasuis* and current vaccines formulated with inactivated strains are capable of inducing limited heterologous protection [3, 4]. To overcome the lack of effective vaccine for *H. parasuis*, management practices and antimicrobial molecules are used to reduce GD outbreaks. However, because of consumers demands for antibiotic-free animal products, public health issues, and growing antibiotic-resistant strains (Miani 2017), the use of antimicrobial molecules might be banned or limited in the future. Thus, an effective vaccine able to induce cross-protection to all heterologous strains is in great demand.

H. parasuis, *Actinobacillus pleuropneumoniae* and *A. suis* are microorganisms armed with membrane proteins able to steal iron from pig's transferrin [5]; these proteins are important virulence factor and essential to bacteria survival in the host [6]. In *H. parasuis*, these proteins are termed transferrin binding protein A (TbpA) and B (TbpB) [7]. TbpA is an integral external membrane protein intimately associated with other receptors TonB-e dependent that are involved with siderophores and vitamin B12 acquisition [8]. TbpA transports iron molecules from the external membrane to the periplasmic space [9]. The second protein, TbpB, is a surface-exposed lipoprotein that independently binds to the host transferrin [5, 10]. Because this iron-capturing complex is central to pathogen survival in the host [11], TbpA and TbpB are potential target antigens for the development of universal vaccines to veterinary [12-14] and human pathogens [9, 15, 16].

Recently, we developed and structurally characterized the TbpB from *H. parasuis*, *A. pleuropneumonia* and *A. suis*, and we demonstrated its immunogenic and protecting potential in pigs experimentally challenged with a lethal dose of *H. parasuis* Nagasaki (SV5) strain [14, 17]. Here, we characterized the functional mechanisms mediated by a panel of antibodies specific to four different forms of recombinant TbpB from the Nagasaki strain of *H. parasuis*

2. Material and Methods

2.1 Expression and purification of recombinant TbpB

The recombinant proteins were expressed and purified essentially as previously described [15]. The gene encoding wild-type TbpB (TbpB-$I^{20\text{-}528aa}$) from *H. parasuis*,

SV 5, Nagasaki strain was cloned into an expression vector and a W176A non-binding mutant was prepared. The mutant gene was then used as a template for amplifying the region encoding the mutant protein (TbpB-$M^{26\text{-}528aa\ [14]}$) or its subdomains (lobes) N (TbpB-$NM^{20\text{-}282aa}$) and C (TbpB-C $C^{283\text{-}528aa}$). These amplified fragments were cloned into the pET20a expression vector resulting in gene encoding a maltose binding protein (MBP)-fusion protein containing a N-terminal polyhistidine tag and a TEV protease cutting site located immediately prior to the TbpB coding region. The expression of the fusion protein was induced by 0.1 mM IPTG (Sigma Aldrich, USA) and the fusion protein purified with a Ni-NTA column (HisTrap™HP, GE Healthcare, USA) followed by cleavage with TEV protease and purification of the TbpB derived protein by ionic exchange (Sepharose-Q, GE, Alemanha).

2.2 Experimental vaccine formulation and swine immunization protocol

Twenty mixed-bred (Large White x Landrace) piglets were used in this experiment. Piglets were deprived from maternal colostrum and kept in a facility room free of specific pathogen (SPF) under biological security level II. Piglets were fed pasteurized cow colostrum and milk followed by commercial feed pellets and were free of anti-*H. parasuis* antibodies. Twenty eight days after birth the piglets were weighed, tagged and divided into five groups (4 piglets/group). The piglets in each group were then immunized with 200 μg of TbpB-I, TbpB-M, TbpB-Nm or TbpB-C, respectively, with Montanide IMS 2215 (VG PR, Seppic, France) as adjuvant in a 2 ml final volume, by the intramuscular route. The fifth, control group was injected with sterile phosphate buffered saline (PBS, pH 7.2) mixed with Montanide (PBS-IMS). All piglets were

immunized again 21 days later with the same antigen formulation. Blood samples were collected prior to and 14 days after each immunization. The experiment followed the guidelines of the Brazilian College of Animal Experimentation and was approved by the institutional Committee for Ethical Use of Animals (protocol nº 015/2014).

2.3 Polyclonal mouse antibody production

Fifty micrograms of purified TbpB-I, TbpB-M, TbpB-NM or TbpB-C with Freund's complete adjuvant (FCA), Montanide IMS 2215 VG PR or Montanide Gel 01 PR (Seppic, France) in 0.5 ml final volume were injected by the intraperitoneal route into two eight-week old mice each. For a negative control, PBS was mixed with each adjuvant and injected into two mice each. Twenty-one days later all mice were re-immunized with the same formulation except mice immunized with FCA; for these mice, the second immunization was carried out using Freund's Incomplete Adjuvant. Blood samples were collected by cardiac puncture from mice anesthetized with Isoflurane (Cristália, Brazil) at fourteen days after the second immunization. After clotting, serum was separated and frozen at -80ºC until usage.

2.4 Enzyme-linked immunosorbent assay to detect specific antibodies in piglets

Each antigen used to immunize piglets (TbpB-I, TbpB-M, TbpB-Nm and TbpB-C) was adsorbed overnight at 4º C in 96 well polystyrene plates (10 μg/well) in a final volume of 100 μl of carbonate buffer (150mM Na_2CO_3, 350mM $NaHCO_3$, 30mM NaN_3, pH 9.6). Non-adsorbed antigen was removed and the wells were blocked with

115 1% bovine serum albumin (BSA) in PBS (150 mM NaCl, 50 mM sodium phosphate,
116 pH 7.2) containing 0.05% Tween-20 (PBS-T). Piglet's serum samples were diluted
117 1:100 in PBS-T and added in duplicates to the wells for 1 h at 37ºC. Then, all wells
118 were rinsed three times with PBS-T and incubated with goat anti-pig IgG peroxidase
119 conjugated (Sigma Aldrich, Brazil) diluted 1:20.000 in PBS-T for and additional 1h at
120 37ºC. After removing the conjugate solution all wells were again rinsed three times
121 with PBS followed by the addition of the peroxidase substrate, tetramethylbenzidine
122 (TMB – Sigma Aldrich, Brazil). After incubation for 10 min at 22º C the plates were
123 read at 450 nm.

124

125 *2.5 Cross-reactivity analysis of serum by flow cytometry*

126 Six reference strains of *H. parasuis* (Nº4 – SV1, SW124 – SV4, Nagasaki –
127 SV5, H425 – SV12, 84-22113 – SV14 and 84-15995 – SV15) were cultivated in
128 *pleuropneumoniae-like organisms* broth (Difco, USA) supplemented with 40 μg/ml of
129 nicotinamide adenine dinucleotide (Sigma Aldrich, Brazil), 2,5 mg/ml of glucose
130 (Sigma Aldrich, Brazil) and 200 μM of 4,4-dipiridile (Sigma Aldrich, Brazil). The
131 bacteria were cultivated under constant rotation (250 rpm) at 37º C for 12 h and then
132 washed twice with PBS and counted. Serum samples from all immunized piglets were
133 heat-treated (56ºC, 30 min) and aliquots of 10 μl were incubated with 1×10^6 bacteria
134 for 1 h at 37ºC. The bacteria were washed three times with PBS and coupled antibodies
135 were detected using fluorescein labelled (FITC) goat anti piglet IgG (AbD Serotec, UK)
136 diluted 1:1000 in PBS 1% BSA for 1 h at 37ºC. The bacteria were then washed again
137 three times with PBS and suspended in 200μl of FACS buffer (PBS+0.5% BSA, pH

7.2) and analyzed by flow cytometry (BD FACSVerse™ – BD Biosciences, USA) equipped with blue and red laser, and volumetric counter. The bacteria population was characterized according to size (forward-angle scatter – FSC), complexity (side-angle scatter – SSC) and green fluorescent emitted by the FITC. The data was analyzed using BD FACSuite™ software (BD Biosciences, USA).

2.6 Isolation of pig peripheral polymorphonuclear (PMN) leukocytes

Blood samples from two pigs were collected using EDTA containing tubes. PMN were isolated using Ficoll-Paque Plus (GE Heathcare, Sweden) following manufacture's instruction, and contaminating erythrocytes were lysed using Red Blood Cell Lysing Buffer (Sigma Aldrich, Brazil). The purity and cell identity was confirmed by flow cytometry taking in consideration the characteristics of the cells (FSC *vs* SSC) and the expression of the antigen recognized by monoclonal antibody (mAb) clone 6D10 isotype IgG2a (kindly provided by Dr. Javier Domíngues Juncal) followed by detection with rat mAb anti-mouse IgG2a biotin-labelled (clone R19-15, BD Pharmingen™, USA) in the presence of SA-APC (BD Pharmingen™, USA). Cell viability was evaluated by propidium iodide (PI, Invitrogen, Brazil) and was always higher than 95%.

2.7 Haemophilus parasuis labelling with FITC

H. parasuis was labelled as previously described Olvera [18] with minor modifications. All strains were grown in PPLO broth to reach OD 0.7 at 600nm (Nanophotometer, Implen, Germany). Bacteria were then washed three times with PBS, counted by flow cytometry and, for labelling, 1×10^9 bacteria/ml in PBS were incubated

with 1 μg of FITC (Sigma Aldrich, Brazil) under agitation (300 rpm) for 30 min at 22 ºC. The labelled bacteria were then washed twice with an excess of PBS to remove unbound FITC and then suspended in 10 ml of PBS containing 5% BSA for 15 min. Labelled bacteria were collected by centrifugation (4.000 × g, 10 min) and suspended in 1 ml of PBS and kept at 4º C.

2.8 Antibody-mediated phagocytosis

The phagocytic assay was performed as previously described by Olvera [18] with minor modifications. First, we defined the piglet's serum dilution. Then, all FITC-labelled bacteria used in this study (strain Nº4, SW124, Nagasaki, H425, 84-22113 and 84-15995) were incubated (1×107) with the polyclonal piglet antibodies at 37ºC for 45 min under constant agitation (300 rpm). The opsonized bacteria were then washed to remove non-coupled antibodies and incubated with 1×10^6 neutrophils cultures in 96 well plates (TPP, Sweden) at 37º C in a final volume of 100 μl of RPMI 1640 medium (Invitrogen, Brazil) supplemented with 10% bovine fetal serum (FBS) (Invitrogen, Brazil) and 100 mM of L-glutamine (Sigma Aldrich, Brazil). After 15 min, the plates were incubated on ice, washed three times with PBS+1% FBS and suspended in 200 μl of PBS+1% FBS. Phagocyting cells were then analyzed by flow cytometry and the total number of neutrophil-associated bacteria was estimated by total fluorescence. In addition, intracellular bacteria were calculated after quenching with trypan blue (31 ug/ml; Sigma Aldrich, Brazil) as previously described by Van Amersfoort and Van Strijp [19].

2.9 Activation of the complement classical pathway and antibody bactericidal activity

In these assays we evaluated the capability of serum from immunized piglets and mice in activating the classical pathway of complement system and to kill *H. parasuis*. All strains of *H. parasuis* were grown in iron-restricted medium, washed twice with buffer CSAB (210 mM triethanolamine, 180 mM citric acid, 10.5 mM MgCl, 1.8 mM CaCl, 1.3 M NaCl, pH 7.4) and counted. Then, 1×10^7 bacteria were mixed with 50 µl of inactivated serum (diluted 1:5 in CSAB), 25 µl of guinea pig complement containing 5 units of C'H50 (see below), in a 96-well plate, and incubated for 1 h at 37ºC. Then, we added 25 µl of the hemolytic system (HS) comprised of sheep red blood cells (SRBC, 2%) sensitized with rabbit hyperimune serum and incubated for an additional hour at 37ºC. The plates were then centrifuged (250 × g for 2 min) and the supernatant were harvested and read at 540 nm for estimate the amount of lysed SRBC.

The reagent used in this assay (rabbit anti-SRBC hyperimmune serum and guinea pig complement) were titrated prior to the experiments. The optimal dilution of rabbit anti-SRBC was 1:1.500 and the dilution of guinea pig complement that gave 50% hemolysis (C'H50) of SRBC was 1:800. And, for the assay, we used 5 units of C'H50. As controls in the complement assay we used: a) positive antibody control [pig hyperimune serum against *E. coli* TOP10 + TOP10 cells + C' + CSAB] and HS: no hemolysis; b) C' control [C' + CSAB] and HS: 100% hemolysis; c) serum nonspecific C' inhibitors [pig serum + C' + CSAB] and HS: 100% hemolysis; d) classical activation pathway control [TOP10 cells + C' + CSAB] and HS: 100% hemolysis; e) hemolysin control [*H. parasuis* + CSAB] and HS: no hemolysis; f) activation buffer control [CSAB only] and HS; no hemolysis.

The assay was interpreted as follows: the presence of SRBC hemolysis, detected by spectrophotometry (540 nm), indicated that the antibodies were unable to activate the complement system. In contrast, the absence of hemolysis indicated that the complement system was activated and was depleted as consequence of antibodies-antigen complex formed in the assay.

The bactericidal assay evaluated the capability of the antibodies to kill the bacteria by activating the complement classical pathway. The essay was similar to that described for complement activation. However, in this assay, we used bacteria (1×10^6) and serum from mice immunized with *H. parasuis*. The reaction consisted of *H. parasuis* (1×10^6) + mice serum + C' + SCAB which was incubated at 37º C for 1h. Then, the non-killed bacteria was detected by chocolate agar and quantitated after 24 h of incubation at 37ºC. Negative control consisted of serum from mice inoculated with PBS.

2.10 Statistical analysis

The data were tested for normality by Kolmogorov-Smirnov and Levene test. Differences amongst treatments were analyzed by the kruskal Wasllys or one-way ANOVA followed by Tukey post test. The results are reported as means ± SEM and *P*-values < 0.05 were considered to be significant.

3. Results

3.1 Recombinant antigen production

The genes encoding TbpB-I, TbpB-M, TbpB-Nm and TbpB-C from *H. parasuis*, SV5, strain Nagasaki were successfully cloned into the expression vector pET20a. The fusion proteins were purified, digested with TEV protease and the TbpB derived proteins were purified and analyzed by standard polyacrylamide gel electrophoresis (SDS-PAGE). The purity of the proteins is illustrated in Figure 1. TbpB-I and TbpB-M are 57 kDa, TbpB-Nm is 31kDa and TbpB-C is 26 kDa.

3.2 Protein immunogenicity in pigs

During the immunization protocol all piglets remained healthy. The antibodies induced by each antigen was measured by and indirect ELISA. The most immunogenic antigen was TbpB-M followed by TbpB-C, TbpB-I and TbpB-Nm, respectively (Figure 2). Specific antibodies were significantly higher ($p < 0.05$) after first and second immunization increasing up to 6 times the initial reading for TbpB-M and TbpB-C and from 2 to 3 times for TbpB-I and TbpB-Nm, respectively. No anti-TbpB antibodies were detected in piglets inoculated with PBS indicating absence of cross-reactivity or co-infection by *H. parasuis*.

246 *3.3 Cross-reactivity profile of piglets' anti-TbpB antiserum towards virulent strains of*
247 *H. parasuis*

248 Anti-TbpB antibodies produced by piglets immunized with the different forms
249 of TbpB were able to recognize four reference strains *H. parasuis* belonging to SV1, 5,
250 12, 14 and 15. In contrast, the same panel of antiserum recognized a smaller percentage
251 of SV4 reference strain (Figure 3A). Nonetheless, the data emphasizes the potential of
252 the antigen (TbpB) in generating antibodies with high cross-reactivity. Antibodies from
253 piglets immunized with TbpB-M recognized on average approximately 72% of bacteria
254 from SV 5, 12, 14 and 15. A smaller percentage (68%) of reference strains were
255 recognized by serum from piglets immunized with TbpM-I, TbpB-Nm and TbpB-C.
256 Interestingly, a small percentage of bacteria belong to SV4 reference strain was
257 recognized by our antiserum panel.

258 Piglets antibodies to TbpB-M, TbpB-Nm and TbpB-C bound with the same
259 efficiency to *H. parasuis* serovars (Fig. 3B), as determined by the mean fluorescence
260 intensity (MFI) on the bacteria surface. In general, the average fluorescence obtained
261 with antibodies to TbpB-I was lower compared to antibodies to the other antigens,
262 except when sorovars 4 and 14 were assayed. The MFI detected on the surface of
263 serovars 1, 4, 5, 12 and 14 was higher when antibodies against TbpB-M were used
264 (table 2). Against SV15 the highest MFI was achieved with the serum from piglets
265 immunized with TbpB-C and for SV1 higher MFI was achieved with anti-TbpB-M
266 antibodies (Table 2).

267

268 *3.4 Antibody mediated phagocytosis*

269 The number of bacteria associated with neutrophils 6D10^{+}, at 15 min after the
270 phagocytic assay, was higher when bacteria was previously opsonized by specific
271 antibodies compared to non-opsonized bacteria. The only exception was observed with
272 the strain SW124 (SV4) in which previous opsonization had no effect on the ability of
273 neutrophils to uptake bacteria. In the absence of opsonizing antibodies, strains Nº4
274 (SV1), Nagasaki (SV5) and 84-22113 (SV14) were less resistant to phagocytosis
275 compared to strains SW124 (SV4), 84-15995 (SV 15) and H425 (SV12) and indicated
276 on Table 1.

277 Opsonizing antibodies were also fundamental to speed up the ability of
278 neutrophils to internalize bacteria. In the presence of opsonized bacteria 72% of
279 neutrophils were found to have internalized bacteria compared to only 36% when non-
280 opsonized bacteria was used in the phagocytic assay (Figure 4A). Interestingly,
281 opsonized and non-opsonized strain H425 (SV12) was found mostly at the neutrophil
282 surface rather than internalized. However, when compared to the control, anti-TbpB
283 antibodies were able to improve in at least four times the number of neutrophils with
284 internalized strain H425 (Figure 4).

285 The type of antibodies also affected the ability of bacteria uptake by neutrophils.
286 The highest fluorescence was observed in neutrophils uptaking bacteria opsonized by
287 anti-TbpB-M antibodies. And, with the exception of bacteria belonging to SV15, this
288 was true for all SV tested in our assay. For SV15, anti-TbpB-C antibodies were better
289 in mediating bacteria uptake (Table 2).

290

291 *3.5 Activation of the complement classical pathway*

292 None of the piglets antibodies against all antigens tested (TbpB-I, TbpB-M,
293 TbpB-Nm and TbpB-C) were capable of inducing complement activation. These results
294 led us to hypothesize that the type of adjuvant used in our experiment (IMS 2215 VG
295 PR) could induce in piglets a subclass of antibody unable to activate complement.

296 To prove our hypothesis, we used the same antigen/adjuvant formulation to
297 immunize mice. Additional groups were immunized with the same antigens mixed to
298 Freund's adjuvant or with Montanide Gel 01. The sera from mice immunized with
299 antigen mixed with Montanide Gel 01 PR and Freund's adjuvant had a similar
300 complement activation profile toward the 6 virulent strains of *H. parasuis* tested. With
301 the exception of anti TbpB-I, all antibodies were capable to induce complete (100%)
302 complement activation against Nº4 (SV1), SW124 (SV4), H425 (SV12), 84-22113
303 (SV14) and and 84-15995 (SV15) strains (table 3). Anti-TbpB-I antibodies induced
304 using Freund and Montanide Gel 01 adjuvants partially activated complement against
305 SV 1 (50% and 20%, respectively), SV 4 (80% both) and SV 5 (50% both) (table 3).
306 And, as observed with pigs, none of the mice immunized with the antigens mixed to
307 Montanide IMS 2215 VG PR were capable of complement activation.

308

309 *3.6 Bactericidal effect of the classical complement activation pathway on Nagasaki*
310 *strain*

311 Sera from all mice immunized with recombinant antigens adjuvanted with
312 Montanide Gel 01 PR) reduced ($p<0.05$) the number of live bacteria (Fig. 6). Antibodies
313 to TbpB-M and TbpB-C reduced bacteria viability by 98% whereas antibodies to TbpB-
314 I and TbpB-Nm inactivated 73% and 45% of the bacteria, respectively (Fig. 6).

315

316 **4. Discussion**

317 The development of a universal vaccine capable of inducing protection to all *H.*
318 *parasuis* serovars remains a major challenge to researchers and would be invaluable to
319 overcome one of the most important bacterial infections of swine herd worldwide.

320 Whole cell based inactivated vaccines are known to be effective against most
321 pathogens that are not prone to superficial antigenic variation. In the other hand, in the
322 case of highly phenotypically variable pathogens such as *H. parasuis*, whole cell
323 vaccines formulated with one or more serovars demonstrate variable degree of
324 protection [3, 4, 20, 21] which might be even reduced by the type of adjuvant used [21].

325 The effectiveness of vaccines based on recombinant proteins relies on the
326 capacity of a given antigen to induce immunity towards pathogens that naturally
327 express that same antigen on its surface. The main challenges for developing such a
328 vaccine depends on the identification of highly conserved antigenic structures that are
329 central to pathogenesis and, in addition, capable of inducing a functional specific
330 immune response able to mediate neutralization, or destruction via phagocytosis, or by
331 mediating activation of the complement classical pathway.

332 The survival of *H. parasuis* in the host depends on the efficient capture of iron
333 molecules from pig transferrin (pTf), mediated by 2 surface proteins, named TbpA and
334 TbpB [22]. Because they are central in H. parasuis pathogenesis, these proteins cannot
335 be lost or significantly altered. Recently, we characterized the H. parasuis TbpB
336 structure and by using a mutant version of the recombinant TbpB we demonstrated its
337 potential as a vaccine candidate [14].

Here, we demonstrate that the C lobe of the TbpB (TbpB-C) protein is highly immunogenic to swine capable of inducing antibodies titers similar to that induced in in pigs immunized with the TbpB-M antigen (Figure 2). This finding suggests that the Tbpb-C contains an epitope repertoire highly recognized by MHC-II molecules, BCR and TCR molecules. Interestingly, TbpB-I (TbpB wild type recombinant), which has in biological function preserved, induced significantly lower antibody titers when compared with TbpB-C, which suggests that binding of pTf to the the N lobe of TbpB could block important antigenic determinants existent in the N and C lobe, hampering clonal selection of B cells, as we recently hypothesized [14, 17]. Because *H. parasuis* heterogeneity, we evaluated the heterologous recognition potential of antiserum produced in swine against serovars 1, 4, 5, 12, 14 and 15, which are known to trigger Glässer Disease [23]. Except SV 4, all serovars were highly recognized by the antisera tested, indicating that the SV 5 TbpB contains antigenic and immunogenic epitopes highly conserved amongst H. parasuis serovars.

None of the antisera (anti-TbpB-I, TbpB-M, TbpB-Nm and TbpB-C) recognized 100% of the bacteria within the bacteria population analyzed in each serovar (Figure 3 C), a phenomenon that could be explained by the presence of endogenous pTf in the pig sera that could have bound to the bacteria and prevented TbpB recognition by antibodies. In addition, we further suggest that pTf binding to TbpB would reduce the number of antibodies molecules that could bind to the bacteria, in that the fluorescent intensity obtained with the antiserum produced towards TbpB-I was slightly lower compared to the fluorescent intensity obtained with the other antiserum. Furthermore, although the titer of antibodies in the antisera against TbpB-M and TbpB-C were higher compared to the antisera against TbpB-I and TbpB-Nm (ELISA data).

Interestingly, although the antibody level against TbpB-M and TbpB-C were higher than those observed in piglets vaccinated with TbpB-I and TbpB-Nm (ELISA data), a proportional antigenic recognition (fluorescent intensity) was not observed on bacteria surface, suggesting that a similar number of epitopes could be being recognized, with the exception of the serum against TbpB-I (figure 3-B).

Because *H. parasuis* is capable of surpassing the host mucosal defenses [24] reaching the bloodstream, we found that the opsonizing capability of the antibodies produced should be evaluated using blood neutrophils, mainly because the implication of this cells on the pathogenesis of Glässer Disease has already been demonstrated [25]. Neutrophils are the major leukocytes found amongst vertebrates and their role on recognition and destruction of bacterial pathogens has been reviewed [26, 27]. Phagocytosis by neutrophils can be mediated by several receptors such as complement (CRs) and immunoglobulins receptors (FcγRII e FcγRIII) [28]. Here, we demonstrate that the number of neutrophils with bacteria is higher in the presence of antibodies (Table 1) and that antibodies also accelerates pathogen engulfment (Figure 4A), highlighting the functional properties of the antibodies induced by the recombinant antigens. A higher number of neutrophil-associated bacteria were observed when we used anti-TbpB-M and anti-TbpB-C antiserum in the assay.

In contrast, we observed that none of the pig antiserum mediated complement activation. Because the type of immune response and immunoglobulin class might be related to the type of adjuvant used in the immunization [29], we hypothesize that the adjuvant Montanide IMS 2215 VG PR could have induced a IgG subclass with reduced capacity to induce complement activation (C'), such as IgG1 [30], induced mostly by IL-10 [31]. Indeed, Martínez-Martínez, Frandoloso [17] found that TbpB adjuvanted

386 with Montanide IMS 2215 increased the transcription of Th2 cytokine, including IL-
387 10.

388 Thus, to demonstrate the role of adjuvants on the type and functionality of the
389 antibodies produced against the recombinant antigens, we immunized mice with the
390 antigen mixed to 3 different adjuvants (Montanide IMS 2215, Montanide Gel 01 and
391 Freund adjuvant). In this experiment we found that the serum of mice immunized with
392 antigen adjuvanted with Montanide IMS 2215 was unable to activate complement.

393 Recently, Xue, Zhao [21], using a trivalent *H. parasuis* bacterin, demonstrated
394 that the type of adjuvant is central in determining a protective immune response and
395 that antibodies titers might not ensure protection. In this scenario, our results contribute
396 to elucidate possible vaccine failure mainly those that could be related to the class and
397 subclass of immunoglobulins induced rather that to the antigen characteristics.

398 In summary, we demonstrated that *H. parasuis* TbpB recombinant proteins are
399 capable of inducing antibodies with a wide range of cross-reactivity within virulent
400 serovars and that conserved epitopes are distributed in both TbpB lobes. Antibodies to
401 TbpB-M and TbpB-C were more capable to mediate bacteria uptake by neutrophils,
402 and these two antigens and the TbpB-Nm mixed with an appropriated adjuvant can
403 mediated the complete activation of the classical pathway of complement system and
404 kill the *H. parasuis* Nagasaki strain.

405 Our results indicate that a vaccine formulated with TbpB-M and/or TbpB-C
406 associated to Montanide Gel 01 (recommended for pigs) could induce antibodies
407 capable of mediating important immune functions that result in a better protection to
408 *H. parasuis* challenge.

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415

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468 bacterial clearance after experimental infection. Veterinary immunology and
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526 **Figures**

527 **Figure 1.** Denaturing SDS-PAGE analysis of purified recombinant TbpB proteins.
528 MW: molecular weight markers; 1: Intact TbpB (TbpB-I); 2: Mutant TbpB (TbpB-M);
529 3: Mutant TbpB N lobe (TbpB-Nm); 4: TbpB C lobe (TbpB-C). The arrows indicate
530 the estimated molecular weight of each protein derived from the amino acid sequence.

531

532 **Figure 2.** Antibody response in pigs immunized with recombinant TbpB-I, TbpB-M,
533 TbpB-Nm and TbpB-C. The level of antibody is indicated as the optical density (OD,
534 450 nm). The data is expressed as the mean (n = 4) ± SEM of each group. The horizontal
535 discontinuous line represents the average OD from all piglets prior to immunization.

536

537 **Figure 3.** Antigenic analysis of antibodies against TbpB-I, TbpB-M, TbpB-Nm and
538 TbpB-C. A) Cross-reactivity intra-species profile of antibodies against virulent strains
539 of *H. parauis*. B) Specific fluorescent intensity from each antiserum group. C) Flow
540 cytometry analysis of *H. parasuis* SV15.. C.i: Flow cytometric Densit Plot (hybrid) of
541 *H. parasuis* (SV15) using side-side scatter (SSC) versus forward-side scatter (FSC)
542 parameters (P1: gate of study). C.ii: *H. parasuis* labeled with serum from piglet
543 inoculated with PBS-IMS. The secondary antibody was goat-anti pig IgG FITC labelled
544 (GAP-IgG-FITC) (negative control). C.iii: bacteria labeled with serum from a piglet
545 inoculated with TbpB-M, followed by GAP-IgG-FITC. Approximately 81% of bacteria
546 population was highly recognized. The statistical comparison was performed amongst
547 the 6 serovar represented in picture A or between the four type of antiserum represented

548 in the picture B. Different letter indicate significant differences ($p<0.05$). Data is
549 expressed as the mean ± SEM.

550

551 **Figure 4.** Flow cytometric analysis of pig neutrophil phagocytosis of live opsonized
552 and no opsonized *H. parasuis*. A) Panel demonstrating phagocytosis of serovars 1, 4,
553 5, 12, 14 and 15 non-opsonized or opsonized with swine antibodies to TbpB-I, TbpB-
554 M, TbpB-Nm or TbpB-C. The clear bars represent the percentile of neutrophils at the
555 P5 region (C.1) with internalized bacteria, and the dark bars represent the percentile of
556 neutrophils with surfaced adhered bacteria. Significant differences ($p < 0.05$) are
557 indicated by an asterisk (*). B) Analysis of the labelling process of *H. parasuis* with
558 FITC. B.1: setting the region (P2) of *H. parasuis* study; B.2: detection of *H. parasuis*
559 labelled with FITC (green fluorescence) at the lower right corner, which represents
560 approximately 99% of bacteria from the P2 region. C) Phagocytosis analysis. C.1:
561 definition of the P5 region for the study of neutrophils based on the FSC and SSC
562 parameters; C.2: demonstration of neutrophils immunostaining with monoclonal
563 antibody (AcMo) 6D10 + rat anti-mouse IgG2a biotin + SA-APC (~ 94% of the cells
564 at the P5 region were neutrophils); C.3 and C.4: neutrophils 15 min after phagocytosis.
565 The cell population at the lower right corner indicate the percentage of neutrophils from
566 the P5 region with associated (at the surface or internalized) *H. parasuis* strains 84-
567 22113 and Nagasaki, respectively (Table 1).

568

569

570 **Figure 5.** Classical complement pathway activation and killing of *H. parasuis* Nagasaki
571 strain by mouse anti-TbpB antibodies. Antibodies anti TbpB-I, TbpB-M, TbpB-Nm
572 and TbpB-C inactivated ~ 73%, ~ 98%, ~ 45% and ~ 98% of the bacteria population in
573 the assay, respectively. Significant differences ($p < 0.01$) are indicated by asterisks (**).

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590 **Tables**

591 **Table 1.** Opsonizing effect of antibodies anti TbpB-I, TbpB-M, TbpB-Nm and TbpB-
592 C towards the virulent strains of *H. parasuis*. The color gradient indicates the higher
593 percentage of phagocytosis for each strain. Different letters indicate significant
594 differences ($p < 0.05$) amongst sera in the same column.

595

596 **Table 2.** Estimation of the number of neutrophil-associated bacteria determined as the
597 mean fluorescence intensity emitted by the FITC-labelled bacteria, after phagocytosis.
598 The decrease in color gradient highlights the antibodies that mediated higher bacteria
599 uptake during phagocytosis. Different letters indicate significant differences ($p < 0.05$)
600 amongst sera in the same column.

601

602 **Table 3.** Activation of classical pathway of complement system by IgGs anti TbpBs
603 produced in mice. Antibodies from mice immunized with TbpB-I, TbpB-M, TbpB-C
604 and TbpB-Nm adjuvanted with Montanide IMS 2215 Vg PR, Montanide Gel 01 and
605 Freund's adjuvant were tested against 6 virulent sorovars of *H. parasuis*. The capacity
606 of these IgGs to activate the complement system is represented as percentage of
607 activation.

Figure 1

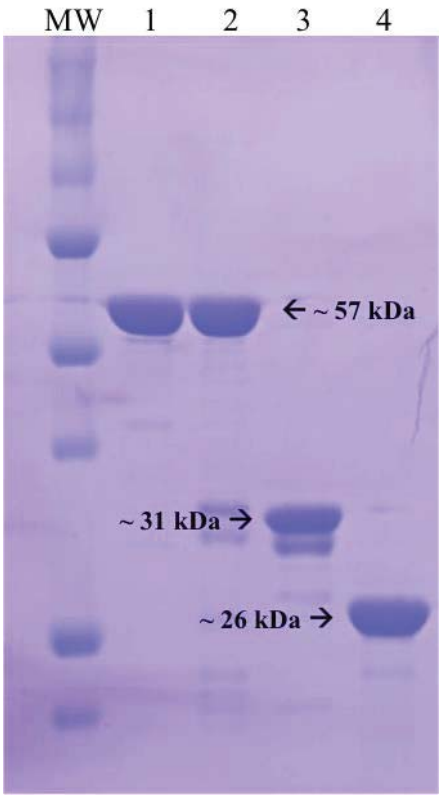

Figure 2

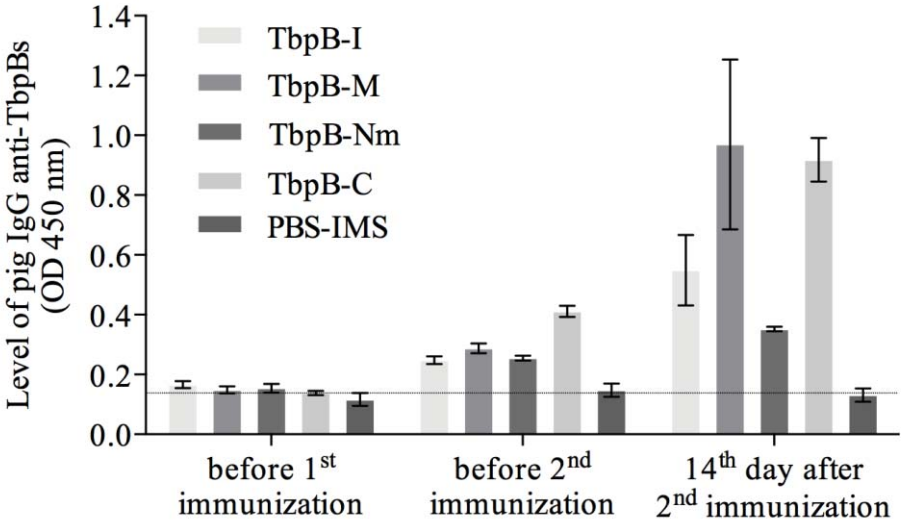
Level of pig IgG anti-TbpBs (OD 450 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
TbpB-I
TbpB-M
TbpB-Nm
TbpB-C
PBS-IMS
before 1st immunization
before 2nd immunization
14th day after 2nd immunization

Figure 3

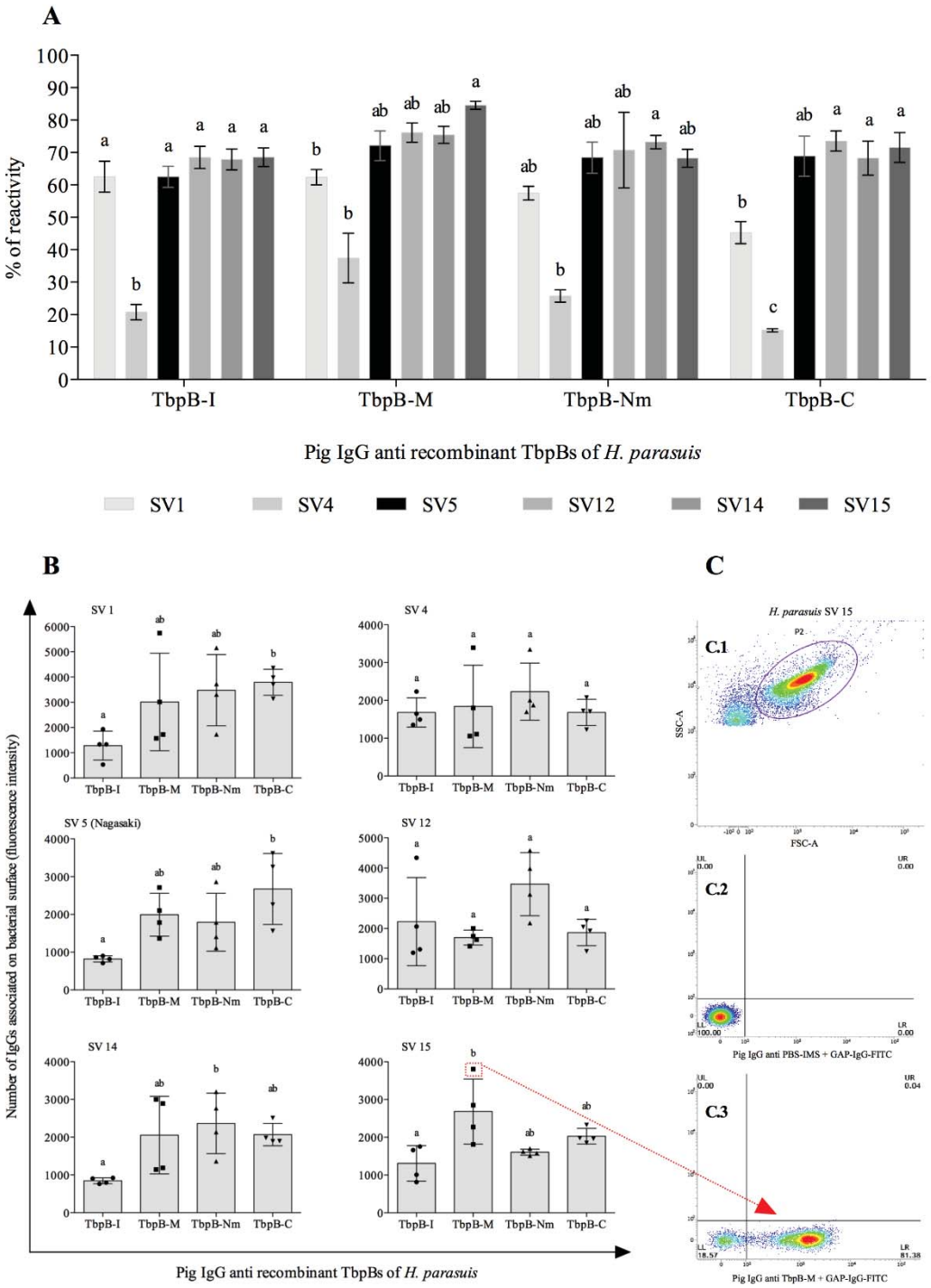
A
% of reactivity
TbpB-I
TbpB-M
TbpB-Nm
TbpB-C
Pig IgG anti recombinant TbpBs of H. parasuis
SV1
SV4
SV5
SV12
SV14
SV15
B
C
SV 1
SV 4
SV 5 (Nagasaki)
SV 12
SV 14
SV 15
Number of IgGs associated on bacterial surface (fluorescence intensity)
Pig IgG anti recombinant TbpBs of H. parasuis
H. parasuis SV 15
C.1
SSC-A
FSC-A
C.2
Pig IgG anti PBS-IMS + GAP-IgG-FITC
C.3
Pig IgG anti TbpB-M + GAP-IgG-FITC

Figure 4

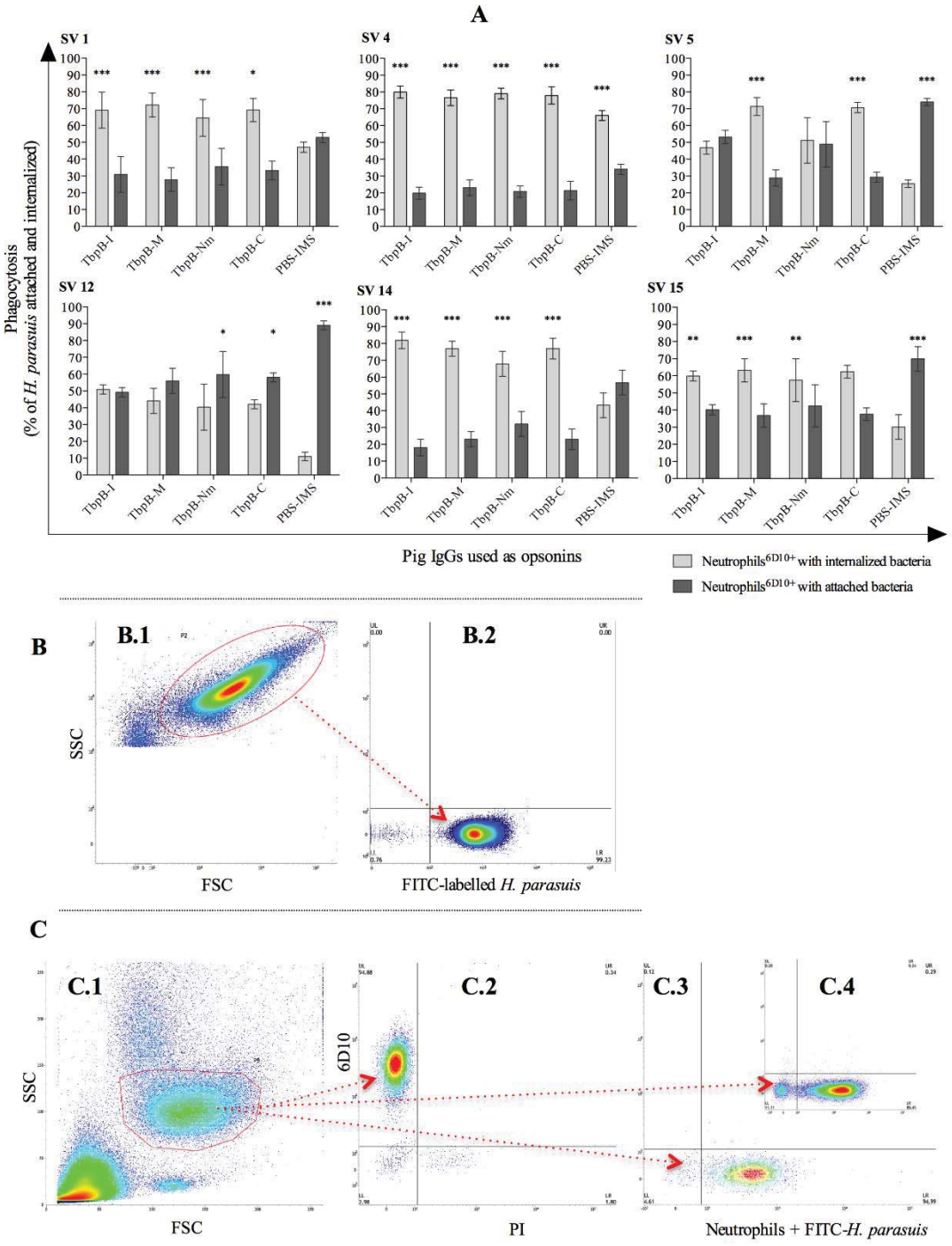
A
SV 1
SV 4
SV 5
SV 12
SV 14
SV 15
Phagocytosis
(% of H. parasuis attached and internalized)
TbpB-I
TbpB-M
TbpB-Nm
TbpB-C
PBS-IMS
Pig IgGs used as opsonins
Neutrophils6D10+ with internalized bacteria
Neutrophils6D10+ with attached bacteria
B
B.1
B.2
SSC
FSC
FITC-labelled H. parasuis
C
C.1
C.2
C.3
C.4
SSC
FSC
6D10
PI
Neutrophils + FITC-H. parasuis

Figure 5

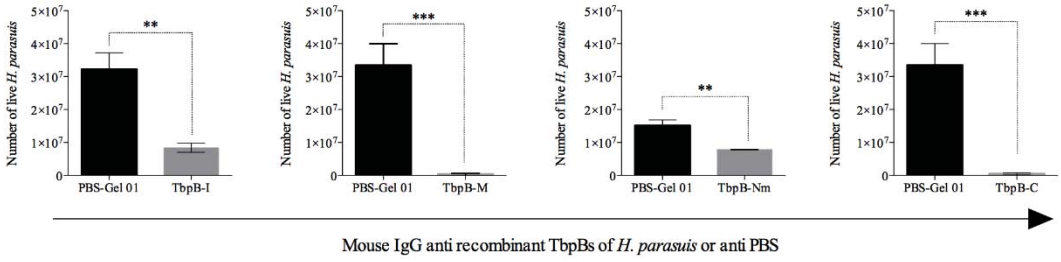
Number of live H. parasuis
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
**
PBS-Gel 01
TbpB-I
***
PBS-Gel 01
TbpB-M
**
PBS-Gel 01
TbpB-Nm
***
PBS-Gel 01
TbpB-C
Mouse IgG anti recombinant TbpBs of H. parasuis or anti PBS

Table 1

Antiserum	% of neutrophils with associated bacteria after 15 minutes of phagocytosis					
	SV1 (Nº4)	SV4 (SW124)	SV5 (Nagasaki)	SV12 (H425)	SV14 (84-22113)	SV15 (84-15995)
TbpB-I	67.6 ± 4.0^{a}	39.9 ± 1.9^{a}	70.0 ± 3.0^{ab}	62.8 ± 0.7^{c}	89.3 ± 1.5^{b}	54.3 ± 4.7^{b}
TbpB-M	67.0 ± 2.3^{a}	43.1 ± 2.9^{a}	78.4 ± 4.2^{bc}	56.0 ± 2.7^{bc}	87.8 ± 1.3^{b}	63.5 ± 2.5^{b}
TbpB-Nm	56.0 ± 4.5^{ab}	43.1 ± 5.7^{a}	67.0 ± 5.8^{ab}	49.2 ± 3.2^{b}	83.8 ± 3.0^{b}	54.5 ± 6.5^{b}
TbpB-C	62.1 ± 2.9^{ab}	38.9 ± 1.1^{a}	86.2 ± 1.0^{c}	52.2 ± 1.6^{b}	91.3 ± 1.3^{b}	57.4 ± 3.0^{b}
PBS-IMS	50.5 ± 2.0^{b}	37.8 ± 1.3^{a}	54.9 ± 1.3^{a}	26.4 ± 1.2^{a}	56.3 ± 4.4^{a}	32.1 ± 2.5^{a}

Table 2

Antiserum	Mean of fluorescence intensity of neutrophils after 15 minutes of phagocytosis					
	SV1 (Nº4)	SV4 (SW124)	SV5 (Nagasaki)	SV12 (H425)	SV14 (84-22113)	SV15 (84-15995)
TbpB-I	1.779 ± 45^{d}	475 ± 20^{a}	5.067 ± 352^{b}	1.194 ± 37^{b}	868 ± 68^{b}	541 ± 19^{ab}
TbpB-M	2.101 ± 77^{c}	519 ± 26^{a}	6.798 ± 128^{b}	1.297 ± 27^{b}	976 ± 40^{ab}	548 ± 28^{ab}
TbpB-Nm	1.353 ± 29^{b}	467 ± 25^{a}	5.005 ± 343^{b}	1.035 ± 101^{ab}	809 ± 76^{ab}	513 ± 41^{ab}
TbpB-C	1.461 ± 35^{b}	514 ± 44^{a}	6.554 ± 204^{b}	1.066 ± 21^{ab}	942 ± 32^{b}	575 ± 18^{b}
PBS-IMS	380 ± 10^{a}	419 ± 12^{a}	2.655 ± 359^{a}	857 ± 82^{a}	668 ± 42^{a}	425 ± 23^{a}

Table 3

Adjuvant	Reference sorovar	Antigen classical pathway activation (%)			
		TbpB-I	TbpB-M	TbpB-C	TbpB-Nm
Montanide IMS 2215 VGPR	1	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	12	0	0	0	0
	14	0	0	0	0
	15	0	0	0	0
Montanide gel	1	20	100	100	100
	4	80	100	100	100
	5	50	100	100	100
	12	0	100	100	100
	14	0	100	100	100
	15	0	100	100	100
Freund	1	50	100	100	100
	4	80	100	100	100
	5	50	100	100	100
	12	0	100	100	100
	14	0	100	100	100
	15	0	100	100	100

4. CONCLUSÕES

Primeira

Os antígenos recombinantes TbpB-I, TbpB-M, TbpB-Nm e TbpB-C potencializados com o adjuvante Montanide IMS 2215 VG PR demonstraram-se imunogênicos em suínos e, antigenicamente, todos os anticorpos específicos foram capazes de reconhecer epítopos nativos presentes na estrutura da TbpB de *H. parasuis*;

Segunda

A expressão homogênea da proteína TbpB nativa de *H. parasuis* durante o cultivo *in vitro* do microrganismo é alcançada somente em condições restritivas de ferro. A inclusão deste protocolo de cultivo durante a produção de antígenos vacinais baseados em corpos celulares inteiros inativados, pode proporcionar melhor cobertura vacinal, em razão das características imunogênicas e antigênicas da proteína TbpB;

Terceira

Os antígenos recombinantes baseados na estrutura da proteína TbpB da cepa Nagasaki de *H. parasuis* induzem anticorpos que reconhecem eficientemente os sorovares 1, 5, 12, 14 e 15 deste microrganismo, destacando o potencial de resposta heteróloga da proteína TbpB;

Quarta

Epitopos antigênicos estão dispostos tanto no lóbulo-N como no lóbulo-C da proteína TbpB nativa de todos os sorovares avaliados. O número de moléculas de anticorpos associadas à superfície do patógeno não difere em relação a especificidade do anticorpo utilizado, indicando um possível bloqueio físico de outros epítopos dispostos na superfície da TbpB quando um ou mais anticorpos se unem ao antígeno. Funcionalmente, um menor número de anticorpos associados às cepas foi observado com o antissoro anti TbpB-I, sugerindo que a transferrina do hospedeiro exerça um efeito negativo sobre a imunogenicidade deste antígeno;

Quinta

Os anticorpos anti TbpB-I, TbpB-M, TbpB-Nm e TbpB-C aumentam a capacidade dos neutrófilos^{6D10+} de capturar de *H. parasuis*, incrementam o número de bactérias fagocitadas, bem como, aceleram o processo de internalização do microrganismo. Entre os antígenos avaliados, destacaram-se as versões TbpB-M e TbpB-C, respectivamente;

Sexta

A susceptibilidade à fagocitose de cepas virulentas de *H. parasuis*, em ausência de anticorpos específicos, varia de acordo com o sorovar. Os sorovares 4, 15 e 12 são naturalmente mais resistente ao processo de fagocitose mediado pelos neutrófilos em comparação com os sorovares 1, 5 e 14;

Sétima

O adjuvante Montanide IMS 2215 VG PR modula a produção de um isotipo de IgG em suínos incapaz de ativar a via clássica do sistema do complemento. Esta limitação funcional pode ser superada com os adjuvantes Montanide Gel 01 e de Freund;

Oitava

Anticorpos gerados em camundongos contra as proteína TbpB-M, TbpB-Nm e TbpB-C foram capazes de ativar eficientemente a via clássica do sistema do complemento contra os todos os sorovares virulentos.

5. CONSIDERAÇÕES FINAIS

Haemophilus parasuis é o agente etiológico da Doença de Glässer, uma doença inflamatória sistêmica, emergente, que produz importantes perdas econômicas durante a produção de suínos.

Hoje, a prevenção das infecções produzidas pelo *H. parasuis* através do uso de vacinas constitui um importante desafio, em razão da alta diversidade antigênica superficial dos diferentes sorovares que produzem doença clínica e, por conta da composição antigênica das atuais vacinas comerciais.

Neste cenário, intensivas práticas de manejo são adotadas para tentar evitar o surgimento da Doença de Glässer, no entanto, na prática, o uso de antimicrobianos é cada vez mais frequente e extensivo durante as fases iniciais da produção de suínos, momento de maior susceptibilidade às infecções produzidas pelo *H. parasuis*.

Neste trabalho, descrevemos a caracterização da resposta imune humoral específica gerada contra quatro antígenos recombinantes desenhados a partir da proteína TbpB de *H. parasuis*. Os resultados apresentados aportam importantes informações funcionais que nos permitem compreender como vacinas produzidas com estes antígenos recombinantes podem neutralização a infecção desencadeada pelo *H. parasuis*.

Os ensaios funcionais padronizados neste trabalho nos permitiram compor uma plataforma *in vitro* de análise e predição de proteção mediada por anticorpos antígenos específicos. Futuros estudos deverão ser delineados para comprovar se a predição dos resultados obtidos *in vitro* se comprovam *in vivo*.

Neste cenário, somos ainda mais otimistas com relação ao potencial de proteção heterólogo de vacinas baseadas em antígenos recombinantes derivados da proteína TbpB. Novos antígenos recombinantes deverão ser projetados para melhor o reconhecimento do SV4, o qual demonstrou-se ser antigenicamente bastante diferente da TbpB do SV5.

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