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João Antônio Guizzo

Streptococcus equi subespécie *equi*: DESENVOLVIMENTO DE UM ELISA INDIRETO E AVALIAÇÃO DA INFLUÊNCIA DO PROCESSAMENTO DO ANTÍGENO NA CAPACIDADE PROTETORA DE BACTERINAS

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária.**

Orientador: Profª. Drª. Agueda Castagna de Vargas

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Aprovado em 10 de março de 2022:

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Santa Maria, RS
2022

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RESUMO

Streptococcus equi subespécie *equi*: DESENVOLVIMENTO DE UM ELISA INDIRETO E AVALIAÇÃO DA INFLUÊNCIA DO PROCESSAMENTO DO ANTÍGENO NA CAPACIDADE PROTETORA DE BACTERINAS

AUTOR: João Antônio Guizzo

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A adenite equina é uma doença altamente contagiosa, de caráter inflamatório e desenvolvimento agudo, que acomete o trato respiratório superior dos equídeos ocasionando grandes perdas econômicas e déficit no desempenho dos animais acometidos. O agente etiológico é o *Streptococcus equi* subesp. *equi* (SEE), o qual possui um variado repertório de fatores de virulência que, em conjunto, propiciam ao microrganismo a capacidade de infectar e causar doença nos equinos. Globalmente, a adenite equina é considerada a doença com maior número de notificações na espécie equina. Portanto, ferramentas que venham somar na prevenção de surtos de adenite equina são extremamente necessárias e alvo de pesquisas em diversos laboratórios. Neste estudo, um dos objetivos foi a padronização de um ELISA para detecção de anticorpos contra SEE. O antígeno utilizado foi extraído da superfície de SEE cultivado em meio líquido. Após o cultivo o meio foi centrifugado e o pellet bacteriano obtido foi solubilizado em uma solução aquosa com 0,025% de desoxicolato de sódio. Após um processo de centrifugação e diálise o antígeno foi utilizado para sensibilização de três distintas placas comerciais próprias para ensaios desta natureza. Foram avaliadas, além da melhor placa, as melhores concentrações de antígeno e diluição dos soros equinos. Após otimização, o ensaio apresentou a seguinte configuração: placas MaxiSorp®, sensibilizadas com 1,2 µg do antígeno extraído, e soros equinos testados na diluição de 1:100. Com estas configurações e um *cutoff* de DO_{450nm} 0.250, o ensaio apresentou 100% de especificidade e 95,9% de sensibilidade. Um segundo objetivo do estudo foi avaliar, em camundongos *Swiss*, a capacidade de 4 bacterinas distintas em induzir imunidade e proteção contra o desafio pelo SEE. Para esse estudo, duas cepas distintas de SEE (ATCC e VE) foram cultivadas e o antígeno bruto (AB) ou processado (AP) produzido com cada cepa, foi utilizado como vacina, resultando em 4 formulações distintas: ATCC AB; ATCC AP; VE AB e VE AP. Quatro grupos de camundongos foram imunizados duas vezes com intervalo de 14 dias com cada uma das formulações e duas semanas após a segunda imunização, os camundongos foram desafiados com o SEE pela via intranasal. Após o desafio experimental, os camundongos foram monitorados observando-se a perda de peso, sobrevivência e colonização da cavidade nasal. Ao final do experimento, 83,3% dos camundongos vacinados com as formulações baseadas no antígeno SEE VE (AB e AP) sobreviveram ao desafio. A proteção nos grupos imunizados com vacinas à base de SEE ATCC variou de acordo com a preparação do antígeno. Com exceção da SEE ATCC AP, as demais formulações resultaram em proteção significativamente ($p<0,05$) superior em relação ao grupo não vacinado. Independentemente da cepa, as vacinas formuladas com AB induziram títulos de IgG elevados em comparação com as vacinas contendo AP. A formulação baseada na cepa SEE VE AB foi significativamente mais imunogênica.

ABSTRACT

S. equi subespécie *equi*: DEVELOPMENT OF AN INDIRECT ELISA AND EVALUATION OF THE INFLUENCE OF ANTIGEN PROCESSING ON THE IMMUNOGENICITY AND PROTECTIVE CAPACITY OF BACTERINS

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ORIENTADORA: Agueda Castagna de Vargas

Equine adenitis is a highly contagious acute inflammatory disease that affects the upper respiratory tract of horses and responsible for large economic losses and deficit in the performance of affected animals. The etiologic agent, *Streptococcus equi* subsp. *equi* (SEE), has a repertoire of virulence factors that combined provide the microorganism with the ability to infect and cause disease in horses. Worldwide, equine adenitis is considered the horse disease with the highest number of notifications. Thus, tools and strategies aimed to improve the prevention of equine adenitis outbreaks are extremely necessary and a matter of investigation in several laboratories worldwide. In this scenario, one of the objectives of this study was the standardization of an ELISA to detect horse antibodies to SEE. The antigen we used was extracted from the surface of SEE cultivated in liquid media. For this purpose, the liquid culture was centrifuged and the bacterial pellet obtained was solubilized in an aqueous solution with 0.025% sodium deoxycholate. After a process of centrifugation and dialysis, the antigen was used to sensitize three different commercial ELISA plates. In addition to the best plate, the best concentrations of antigen and dilution of equine sera were also evaluated. Once optimized, the assay had the following configuration: MaxiSorp® plates, sensitized with 1.2 µg of the extracted antigen, and equine sera tested at a dilution of 1:100. With these settings and a cutoff of OD_{450nm} 0.250, the assay had 100% specificity and 95.9% sensitivity. A second objective of this study was to evaluate in Swiss mice the immunogenicity and protecting capability of 4 different SEE bacterin. For this purpose, two strains of SEE (ATCC and VE) were cultivated and the raw (RA) or processed antigen (PA) was used to formulate four distinct vaccines: ATCC RA, ATCC PA, VE RA and VE PA which were inoculated in mice twice, 14 days apart. Two weeks after the second immunization the mice were challenged intranasally. After the experimental challenge, clinical signs, weight loss, survival and nasal cavity colonization were monitored. At the end of the experiment, 83.3% of the mice vaccinated with formulations based on SEE VE (RA and PA) survived the challenge. Protection in groups immunized with SEE ATCC vaccines varied according to the antigen formulation. With the exception of SEE ATCC PA, all formulations resulted in significantly ($p<0.05$) higher protection compared to the unvaccinated group. Regardless of the SEE strain, vaccines formulated with RA induced higher IgG titers compared to vaccines containing PA. Of the 4 vaccines developed, the formulation based on the SEE VE RA strain was significantly more immunogenic.

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1 INTRODUÇÃO

Entre os animais de alto valor zootécnico, os equinos são os que podem ser mais facilmente expostos a doenças infecciosas, uma vez que são frequentemente transportados/deslocados para a participação em feiras, eventos esportivos e comerciais. Essa movimentação propicia o contato entre equinos oriundos de diferentes planteis e regiões geográficas, os quais podem ser portadores de diversos agentes patogênicos (MITCHEL et al., 2021). Com isso, o controle de enfermidades infecciosas de equinos muitas vezes é difícil e altamente dependente de uma imunização e métodos de diagnóstico eficazes.

Dentre as possíveis doenças que acometem equinos merece destaque a adenite equina também conhecido no Brasil como Garrotilho e mundialmente pelo termo de língua inglesa *Strangles*. Apesar dos primeiros relatos datarem de mais de 700 anos (RUFFUS, 1251), o Garrotilho ainda causas grandes prejuízos ao desempenho e bem-estar dos equinos, com consequências econômicas expressivas para a cadeia produtiva (HARRIS et al., 2015); só no Reino Unido são estimados 600 novos surtos de garrotilho todo o ano (PARKINSO; NEWTON, 2010)

O *Streptococcus equi* subespécie *equi* (SEE) é o agente etiológico da adenite equina e está disseminado mundialmente, com exceção da Islândia. Devido a proibição da importação de equinos para o país estar proibida há mais de 1000 anos, o rebanho islandês é o único reduto de equinos livres deste microrganismo e, consequentemente, sem relatos de garrotilho até o momento (BJÖRNSDÓTTIR et al., 2017). A avaliação de genomas de 225 isolados atuais de SEE demonstrou haver 4 clusters distribuídos mundialmente e que aparentemente houve uma redistribuição de cepas do SEE entre o final do século XIX e início do século XX (HARRIS.et al., 2015). Este evento de substituição populacional de SEE foi relacionado à Primeira Guerra Mundial, momento em que equinos de diferentes continentes se encontraram nos campos de batalha. A mortalidade massiva de equinos neste período histórico forçou a criação de grandes centros de reprodução que subsidiaram o repovoamento dos rebanhos mundiais no pós-guerra, fato que contribuiu para redistribuição e baixa variabilidade genômica observado hoje (HARRIS, et al., 2015)

Apesar dos genomas de forma geral apresentarem homologia, alterações pontuais como aquelas que ocorrem no gene *seM*, que codifica a proteína M (considerado o principal fator de virulência de SEE), são observados com maior frequência (CHANTER et al., 2000; KELLY et al., 2006; LIBARDONI et al., 2013). Atualmente existem 242 alelos da SeM mapeados e agrupados em uma plataforma de dados pública (http://pubmlst.org/szooepidemicus/seM/); no

Brasil, somente no estado do Rio Grande do Sul foram descritos 15 distintos alelos (LIBARDONI et al., 2013). As variações na SeM estão localizadas na porção n-terminal e foram relacionadas a um mecanismo de escape do sistema imune; ou seja, que ocorrem com a finalidade de não serem reconhecidas pelos anticorpos (CHANTER et al., 2000). No entanto, até o momento, não existem dados que comprovem que os alelos exerçam variação na reatividade cruzada de anticorpos em cepas classificadas como distintas de acordo com a porção n-terminal da SeM. Timoney et al. (2010), demonstraram não existirem diferenças de reatividade cruzada de anticorpos, assim como não foi encontrada nenhuma variação funcional entre os alelos da SeM testados. Do ponto de vista imunológico e de reatividade cruzada de anticorpos, sejam eles induzidos através de imunizações ou infecções naturais, o SEE aparentemente não pode ser dividido em distintos sorogrupos e apresenta homogeneidade (BAZELEY, 1942; GALÁN; TIMONEY, 1998; MORAES et al., 2009)

Globalmente, existe uma grande variedade de vacinas disponíveis que compreendem bacterinas, vacinas vivas atenuadas e de subunidades (BOYLE et al., 2018). Porém, a utilização de vacinas comerciais convencionais como ferramenta profilática, apesar de diminuir o número de animais infectados em surtos e amenizar os sinais clínicos decorrentes da infecção, está longe de ser uma estratégia que proporcione amplo espectro de proteção. A homogeneidade genômica e sorológica apresentadas pelos distintos isolados de SEE reportados em diferentes estudos, forçam a pensar que talvez os episódios de falhas vacinais possam estar associados a outros fatores, que não a ausência de reconhecimento de epítopos na superfície de SEE pelos anticorpos induzidos. Dentre estes fatores podemos sugerir: *i)* falhas de potência das vacinas, diretamente traduzidas em baixos títulos de anticorpos; *ii)* avidez dos anticorpos induzidos *iii)* conservação da antigenicidade da unidade antigênica após o processamento do antígeno e formulação. Vale ressaltar que no Brasil a única plataforma de vacinas contra a adenite equina disponível são as bacterinas formuladas a partir de células inteiras inativadas de SEE. Porém, apesar da ampla utilização destas vacinas, surtos de adenite equina continuam acontecendo mesmo em rebanhos vacinados.

Com o intuito de contribuir para o desenvolvimento de novas estratégias de diagnóstico e profilaxia, neste estudo apresentamos o desenvolvimento e avaliação de um ELISA indireto, baseado em antígenos de superfícies de *S. equi* subsp. *equi* e também a avaliação do impacto da metodologia aplicada na produção da unidade antigênica de bacterinas contra o garrotilho, sobre a imunogenicidade e capacidade protetora destas vacinas em um modelo murino.

2 REVISÃO BIBLIOGRÁFICA

2.1 ADENITE EQUINA

A adenite equina, também conhecida como Garrotilho, é uma das primeiras doenças de equinos relatadas cientificamente; a infecção possui caráter enzoótico, é de rápida disseminação dentro do plantel equino e acomete preferencialmente animais jovens (TODD, 1910). Apesar ser conhecida e bem caracterizada do ponto de vista epidemiológico e microbiológico a mais de um século (SCHÜTZ, 1888), a adenite equina continua sendo de extrema importância na equinocultura moderna, aparecendo em primeiro lugar em número de notificações dentre as enfermidades que acometem equinos (HARRIS et al., 2015).

A adenite equina é uma doença altamente contagiosa, de caráter inflamatório e desenvolvimento agudo, que acomete o trato respiratório superior dos equinos (WALLER, 2014). A infecção causa grande desconforto devido à febre e ao aumento de volume dos linfonodos retrofaríngeos e submandibulares que obstruem a faringe, e à descarga nasal mucopurulenta, sintomas que dificultam sobremaneira a respiração e deglutição, e que persistem durante semanas (BOYLE et al., 2018). A morbidade é altamente variável, porém em casos de surtos pode acometer 100% do plantel (RIIHIMÄKI et al., 2018). Quando comparada à morbidade, a mortalidade é pouco expressiva: em casos nos quais a doença foi precocemente diagnosticada e tratada de forma assertiva, os índices de mortalidade não costumam ser maiores que 2% (TIMONEY, 1993). Existem relatos de surtos onde o tratamento não foi adotado, ou o diagnóstico feito tardiamente, em que a mortalidade chegou aos 10% (TIMONEY, 2013)

O agente etiológico da adenite equina é o *S. equi* subespécie *equi* (SEE), uma bactéria Gram positiva, β-hemolítica do grupo C de Lancefield (KUWAMOTO et al., 2001). Os primeiros indícios de que um microrganismo em formato de “coco” seria o agente causador do garrotilho datam de 1873, quando Rivolta observou cadeias de cocos no pus proveniente de abcessos de animais acometidos (TODD, 1910). Em 1888, Schütz descreveu com mais detalhes este microrganismo, inclusive sua capacidade de provocar doença similar em camundongos; para isso, os camundongos foram inoculados com pus, contendo cocos, oriundo de equinos que cursavam com sinais clínicos de garrotilho, seguida de um minucioso acompanhamento dos sinais clínicos e isolamento do agente de diferentes sítios sistêmicos dos camundongos

(SCHÜTZ, 1888). Este modelo animal até hoje é utilizado para reprodução da infecção com *S. equi* subespécie *equi*, principalmente em testes de formulações vacinais.

A porta de entrada do agente são as vias oral e/ou nasal, com posterior aderência nas células do trato respiratório superior e cavidade oral, principalmente na região das tonsilas (TIMONEY e KUMAR, 2008). A superfície do tecido tonsilar, tanto do trato respiratório superior quanto da cavidade oral, é um sítio do qual são isoladas grandes quantidades de *S.* equi, o que contrasta com a pequena quantidade deste microrganismo visualizado nas partes mais profundas deste tecido, indicando que uma pequena quantidade de células bacterianas é necessária para infecção (TIMONEY e KUMAR, 2008).

O tecido tonsilar é organizado em folículos no qual estão presentes principalmente linfócitos e células fagocíticas, e tem ligação direta com a circulação linfática (TIMONEY e KUMAR, 2008), o que faz com que em poucas horas (1-3 h) o microrganismo chegue os linfonodos regionais, local aonde ocorre a replicação. Acredita-se que além do transporte do microrganismo livre pela circulação linfática, o *S. equi* subesp. *equi* chegue aos linfonodos regionais associados a células fagocíticas que falharam no processo de inativação do patógeno. Talvez esta seja a principal forma de passagem do tecido tonsilar para os linfonodos, justificada pelo fato de que cepas acapsuladas e com baixa expressão de proteína M, estruturas que conferem resistência a fagocitose, falham no processo de infecção (TIMONEY e KUMAR, 2008). A presença de *S. equi* subesp. *equi* nos tecidos envolvidos promove aumento da permeabilidade vascular, quimiotaxia de células inflamatórias, edema e formação de abcessos com acúmulo de secreção purulenta, que pode levar a abscedação dos linfonodos e a formação de descarga nasal mucopurulenta (MALLICOTE, 2015; TIMONEY e KUMAR, 2008).

O período de incubação *S. equi* subesp. *equi* varia entre 3 e 14 dias contados do momento da exposição. Os sinais clínicos clássicos da doença são febres seguida de linfoadenopatia e sinais de depressão e inapetência, os quais são comuns logo no início do processo infeccioso e estão relacionados diretamente a febre e aumento dos linfonodos (BOYLE et al., 2018). Os linfonodos submandibulares e retrofaríngeos são os mais comumente afetados, no entanto, qualquer linfonodo da região da cabeça e pescoço pode ser colonizado por *S. equi* subesp. *equi* (MALLICOTE, 2015).

Quando o tratamento é instituído tardiamente ou não é realizado, a linfoadenopatia pode progredir com aumento da quantidade de conteúdo purulento no interior dos linfonodos acometidos e possível ruptura e drenagem deste conteúdo. A maturação e drenagem dos linfonodos retrofaríngeos (devido à localização anatômica) pode provocar extravasamento do conteúdo purulento para a nasofaringe, causando descarga nasal, e ou para o interior das bolsas

guturais, causando o empiema das mesmas. O curso da enfermidade está relacionado ao início do tratamento, o qual é contraindicado após o enfartamento dos linfonodos. Quando o tratamento não for instituído, o período de duração fica entre duas a quatro semanas, com recuperação espontânea na grande maioria dos casos após a drenagem do conteúdo dos linfonodos (MALLICOTE, 2015).

Relatos científicos apontam que, mesmo após cessados os sinais clínicos, cerca de 10% dos animais que tiveram formação de abcessos com empiema das bolsas guturais durante o período de infecção, não eliminam completamente o pus das bolsas guturais e das cavidades sinusais (PRINGLE et al., 2019). O pus remanescente com o passar do tempo tende a secar e formar massas sólidas de formato arredondado, denominadas condroides. Associado a estas estruturas, o *Streptococcus* subesp. *equi* assume capacidade de permanecer dentro das bolsas guturais por período indeterminado, tornando o animal um portador assintomático (NEWTON; WOOD; CHANTER, 1997; WOOD et al., 1993).

Os equinos portadores eliminam de maneira intermitente o microrganismo, podendo infectar outros animais do plantel (PRINGLE et al., 2019). A capacidade de *S. equi* subesp. *equi* de estabelecer infecção persistente é um ponto crítico para a alta incidência da doença em nível mundial (WALLER, 2014). Libardoni et al., (2016) em um inquérito epidemiológico realizado em conjunto com a Secretaria da Agricultura, Pecuária e Desenvolvimento Rural (SEAPA-RS) no estado do Rio Grande do Sul, apontaram uma prevalência de 5,86% de equinos portadores em nível de rebanho (LIBARDONI et al., 2016). Muito provavelmente os valores sejam superiores ao apontado, principalmente devido a eliminação de *S. equi equi* por equinos portadores ocorrer de forma intermitente e em pequenas quantidades. Dessa forma, para uma correta determinação da prevalência de portadores, seria indicado pelo menos três coletas de cada animal, com intervalos mínimos de uma semana entre as coletas, e a utilização de métodos moleculares como a PCR em vez de isolamento para a detecção de portadores (WALLER, 2013).

2.1.1 *Streptococcus equi* subesp. *equi*

Estudos demonstram que *S. equi* subesp. *equi* se originou de *Streptococcus zooepidemicus*, outro patógeno de equinos (CHANTER et al., 1997; JORM et al., 1994), os quais possuem 78,2 % de genes ortólogos (HOLDEN et al., 2009). Dessa forma, os dois patógenos estão agora agrupados em uma mesma espécie e divididos por subespécies (RICHARDS et al., 2014).

Por muito tempo, e ainda hoje, na maioria dos laboratórios, a diferenciação entre *S. equi* e *S. zooepidemicus* é feita através da caracterização do perfil fenotípico do isolado, através da capacidade de fermentação de diferentes açúcares. Distintivamente, o *S. equi* não possui habilidade de fermentar lactose, ribose, sorbitol e trealose, enquanto o *S. zooepidemicus*, destes quatro açúcares, só não fermenta a trealose (KUWAMOTO, ANZAI e WADA, 2001). Porém, por mais de uma vez, foram relatados isolados de *S. equi* com perfis de fermentação atípicos (GRANT, EFSTRATIOU e CHANTER, 1993; WOOLCOCK, J. B., 1975; TASCA, 2018), indicando que resultados de diferenciação entre as duas subespécies obtidos através do perfil fenotípico podem estar equivocados.

O *S. equi* subesp. *equi* possui um variado repertório de fatores de virulência que, combinados, propiciam ao microrganismo a capacidade de infectar e causar doença nos equinos. Dentre esses destacam-se a cápsula de ácido hialurônico, as enzimas estreptolisina, estreptoquinase S, fosfolipase A2 (LÓPEZ-ÁLVAREZ et al., 2017). Destaca-se ainda a proteína M, de 58 kDa, codificada pelo gene *seM*, caracterizada pela primeira vez por Galant e Timoney (1987), a qual apresenta aspecto de fimbria e está ancorada na parede celular e se estende através da cápsula de ácido hialurônico ficando exposta na superfície do microrganismo(GALANT; TIMONEY, 1987; BOSCHWITZ; TIMONEY, 1993).

A proteína M desempenha importante papel na patogenia da adenite equina, com funções de aderência às células do trato respiratório e atividade antifagocítica, mediada pela inibição da deposição das opsoninas C3b do sistema do complemento e imunoglobulinas G (BOSCHWITZ; TIMONEY, 1993). A proteína M é responsável também pela deposição de fibrinogênio sobre o microrganismo, que serve como um mecanismo de evasão do sistema imune do hospedeiro (TIMONEY; ARTIUSHIN; BOSCHWITZ, 1997).

Outra característica importante da proteína M de *S. equi* subesp. *equi* é a imunogenicidade. Tanto em animais vacinados com bacterinas quanto em animais infectados naturalmente, há o desenvolvimento de uma robusta resposta de anticorpos contra a proteína M detectada na mucosa do trato respiratório ou no sangue (SHEORAN et al.,1997).

A capacidade desta proteína, na forma purificada, de induzir resposta imune protetora, foi acessada com sucesso por Meehan, Nowlan e Owen (1998) em um modelo experimental baseado em camundongos (MEEHAN; NOWLAN; OWEN, 1998). Em contradição a isso, quando outro grupo de pesquisadores imunizou equinos com uma vacina baseada em epítopos imunodominantes da proteína M associados a uma molécula de cólera toxina, os resultados não foram os satisfatórios (SHEORAN; ARTIUSHIN; TIMONEY, 2002).

Por muitos anos acreditou-se que a sequência do gene *seM* era altamente conservada entre os diferentes isolados de *S. equi* subesp. *equi* (GALANT; TIMONEY, 1987). No entanto, variações no gene e, consequentemente, na proteína, foram descritos inicialmente por Chanter et al. (2000), que identificaram truncamentos na porção N-terminal. Estão disponíveis em um banco de dados online (http://pubmlst.org/szooepidemicus/seM/) 242 distintos alelos da proteína M encontrados (acessado em 15/02/2022). Uma das hipóteses para esta grande variabilidade é a pressão seletiva do sistema imune do hospedeiro (CHANTER et al., 2000; IVENS et al., 2011) demonstrando que a proteína M é um dos principais alvos do sistema imune de equinos.

Baseado na variabilidade da porção N-terminal da proteína M foram desenvolvidos estudos que demostram o potencial desta região na avaliação da epidemiologia de surtos de adenite equina (KELLY et al., 2006). Parkinson et al., (2011) levantam a possibilidade de que mutações no gene *seM* podem levar ao surgimento de novos alelos geograficamente relacionados. Em um trabalho realizado no estado do Rio Grande do Sul, foram encontrados 15 distintos alelos circulantes em populações de equinos de diferentes regiões (LIBARDONI et al., 2013).

2.1.2 Profilaxia da adenite equina

No cenário atual, a profilaxia da adenite equina está baseada em práticas que garantam um bom nível de biossegurança nos criatórios de equinos, como reduzido fluxo de animais, introdução de novos animais precedida de quarentena, isolamento seguido de tratamento de equinos com sinais clínicos da doença, exames clínicos e laboratoriais rotineiros no plantel, e vacinação (WALLER, 2014). Devido as características epidemiológicas e de transmissão do garrotilho, associadas ao perfil de trânsito dos equinos, um protocolo eficiente de vacinação consiste na melhor estratégia para a proteção dos animais contra esta enfermidade (BOYLE et al., 2018)

Globalmente, existem um variado repertório de vacinas contra o garrotilho, que se valem das mais diversas formulações antigênicas. Estão à disposição dos criadores e veterinários vacinas vivas atenuadas, vacinas de subunidades (BOYLE et al., 2018), e também as clássicas bacterinas que são as vacinas produzidas a partir de células bacterianas inteiras de SEE. Estas últimas, quando feitas de forma customizada para determinado plantel, são classificadas como vacinas autógenas. A pesar destas várias formulações estarem a disposição no mercado mundial, no Brasil as possibilidades estão reduzidas às bacterinas, sendo elas:

Vacina Contra o Garrotilho (Ceva Saúde Animal, Campinas, São Paulo, BR), Vacina Inativada Contra o Garrotilho Labovet (Labovet Produtos Veterinários, Feira de Santana, Bahia, BR).

A utilização de cepas vivas atenuadas para imunização encontra suporte principalmente na imunidade induzida por infecções naturais e, no que tange *S. equi*, a imunidade pós infecção é robusta e duradoura (SHEROAN et al., 1997). Até o momento duas vacinas que se valem de cepas vivas atenuadas foram comercializadas para a prevenção da adenite equina. Uma delas, denominada Equilis StrepE, utiliza a cepa TW928, criada a partir da cepa de campo TW, que foi isolada de um linfonodo abcedado de um potro com sinais clínicos de garrotilho na Holanda. A atenuação da cepa TW foi feita através de deleção no gene *aroA* (KELLY et al., 2006) relacionado ao metabolismo celular de SEE utilizando a técnica de "*electroporation of gene knock-out*" (JACOBS, 2000). A cepa Pinnacle, que dá o nome à outra vacina viva atenuada comercializada, também foi criada a partir de mudanças no genoma de SEE. Desta vez o gene alvo foi o gene *hasA* (Hialuronato sintase), um dos três genes que compõem o operon *has* responsável pela produção da cápsula de ácido hialurônico em SEE (WALKER; TIMONEY, 2002). Para obter a atenuação, foi integrado ao gene *has* um fragmento de DNA denominado Hasdel; a inserção deste fragmento fez com que a cepa Pinnacle não fosse mais capaz de produzir a capsula de ácido hialurônico e sua virulência foi atenuada (WALKER; TIMONEY, 2002).

Apesar das vacinas com cepas vivas atenuadas contra a adenite equina supostamente induzem uma imunidade robusta e duradoura, pouco tempo após os estudos iniciais alguns inconvenientes começam a ser apontados. Tanto para a Equilis StrepE quanto para a Pinnacle, existem relatos de efeitos adversos decorrentes da aplicação da vacina. A cepa TW 928 foi isolada de abcessos formados no local de aplicação da vacina (JACOBS, 2000) e também associada a sinais clínicos de garrotilho em animais recém vacinados (KEMP-SIMONDS et al., 2007). Além dos efeitos adversos, outro ponto negativo é a curta duração da imunidade que perdura por cerca de 3 meses após a vacinação com a Equilis StrepE (WALLER; JOLLEY, 2007). A Pinnacle é uma vacina de administração intranasal (instilada), com utilização recomendada somente em animais hígidos com idade superior a 1 ano. Ainda, não é recomendado a administração associada a nenhum outro procedimento invasivo e até mesmo a outras vacinações e administrações medicamentosas (BOYLE et al., 2018). Todas essas recomendações estão relacionadas a um número significativo de relatos de garrotilho após a utilização desta vacina. Aparentemente, a falta de capacidade de produzir a cápsula de ácido hialurônico não parece suficiente para atenuação, uma vez que, mesmo mantendo as mutações, foi isolada de equinos após a vacinação (CURSONS et al., 2015).

No mercado americano é comercializada a Strepvax II, uma vacina cuja a unidade antigênica é composta unicamente pela proteína M (SeM) de *S. equi* subesp. *equi* extraída de células inteiras do microrganismo e posteriormente purificada. Apesar da utilização da SeM induzir altas quantidades de anticorpos já após a primeira vacinação em equinos, os resultados obtidos em experimentos controlados apontam para uma reduzida capacidade protetora da resposta imune induzida por este antígeno (SHEROAN et al., 2002)

Em outubro de 2021 outra vacina de subunidade foi aprovada, desta vez para uso na União Europeia. Trata-se de uma vacina que contém em sua formulação 3 quimeras, que no total somam 8 diferentes antígenos nativos fusionados de *S. equi* subesp. *equi*, produzidos de forma recombinante. Esta vacina foi testada pela primeira vez com a denominação de Strangvac 4, em um experimento aonde mais 4 formulações que continham estas 3 e algumas outras quimeras foram avaliadas (ROBINSON et al., 2017). Devido ao desempenho superior desta formulação, um novo estudo com três diferentes protocolos de vacinação seguido de desafio foi realizado. Os resultados apresentados demostraram uma diminuição dos sinais clínicos nos animais que receberam a Strangvac quando comparados ao placebo. No entanto, em um protocolo de 2 doses, com intervalo de 28 dias entre doses, seguido de um desafio 14 dias após a segunda imunização, 14 de 16 animais vacinados desenvolveram abcessos em pelo menos um linfonodo e, destes 16, apenas 5 puderam ser mantidos até o final do período de avaliação. Submetidos ao mesmo protocolo vacinal, outro grupo de 12 animais vacinados e desafiados 60 dias após a segunda dose, 6 de 12 animais apresentaram abcessos em pelo menos 1 linfonodo. Em um terceiro protocolo, com 2 doses sob o mesmo regime de aplicação das anteriores seguidas de uma terceira dose 3 meses após a segunda imunização, e desafiados 14 dias após esta última dose, 14 de 15 animais desenvolveram abcessos. Outros parâmetros foram avaliados, tais como temperatura retal, tempo até apresentação de febre, concentração de fibrinogênio plasmático e contagem de neutrófilos no sangue (ROBINSON et al., 2020). Aparentemente, apesar da Strangvac ter apresentado excelentes resultados de proteção cínica, com diferenças significativas na temperatura retal, concentração de fibrinogênio plasmático e contagem de neutrófilos no sangue (ROBINSON et al., 2020), não impede que SEE atinja os linfonodos e desenvolva abscessos.

Vacinas que utilizam células inteiras inativadas são amplamente e de longa data empregadas mundialmente no controle de infecções bacterianas e virais (PLOTKIN, 2005); ademais, são a única plataforma de vacinas contra o garrotilho, comerciais ou autógenas, disponível no Brasil. Apesar disso estudos que auxiliem a compreensão e apresentem alternativas para a melhora de performance de bacterinas contra o garrotilho são escassos. As

publicações mais recentes que tratam da vacinação contra a adenite (WALLER; JOLLEY, 2007) utilizam dados muito antigos (JORM, 1990; TIMONEY; EGGERS, 1985) para basear o desempenho das bacterinas, sempre apresentando dados de proteção restritos e ligando a utilização destas vacinas a reações locais. No entanto, dados produzidos por grupos de pesquisa brasileiros, incluindo os apresentados nesta tese, demonstram haver diferentes maneiras de aumentar a performance das bacterinas contra o garrotilho (ROSA et al., 2021).

Na profilaxia vacinal, além da necessidade da utilização de formulações vacinais com capacidade de indução de respostas imunes protetoras, são necessárias ferramentas que possibilitem a avaliação da potência e duração da imunidade induzida pela vacinação. Contar com ensaios que possibilitem estas avaliações é de primeira importância no delineamento de um programa de vacinação; além disso, isso permite posicionar as vacinações visando diminuir a janela imunológica de potros, bem como fazer estímulos de imunidade em momentos oportunos em animais adultos. Para isso, os testes sorológicos são necessários e os ensaios do tipo ELISAs são os mais indicados.

Atualmente, existem 3 testes comerciais de ELISA para detecção de anticorpos contra SEE: dois destes utilizam a SeM como único antígeno (BOYLE et al., 2018) e um terceiro, um duplo iELISA, utiliza como antígeno as porções n-terminais da SeM e da SEQ2190 (ROBINSON et al., 2013). Mais uma vez no Brasil não contamos com nenhum destes testes de ELISA aprovados para comercialização, o que nos deixa dependente de ensaios desenvolvidos *in house*.

3 MANUSCRITO 1

Development of an indirect ELISA based on surface antigens of *Streptococcus equi* subsp. *equi*

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Abstract

Strangles is the equine disease most reported worldwide. It is a highly contagious infectious disease caused by *Streptococcus equi* subesp. *equi*. Serological diagnosis assays are useful tools that help monitoring the disease spread and mitigate outbreaks by assessments of herd immunity. In the present study, we designed an indirect ELISA (iELISA) based on proteins extracted from the *Streptococcus equi* subesp. *equi* surface. We evaluated protein concentration, type of ELISA plate and horse serum dilution to optimize the assay. The MaxiSorp® and Microlon®600 plates did not differ from each other in terms of antigen adsorption. However MaxiSorp® plate coated with SEE-DOC 1.2 µg/well, and horse serum diluted 1:100 were the choices during the standardization. With the cutoff point set at 0.250 (OD_{450nm}), using sera from pre-suckle foals (negative) and from convalescent/vaccinated (positive) horses, the sensitivity and specificity estimates were 100% and 95.9% respectively.

1 Introduction

Streptococcus equi subsp. *equi* (SEE) is the etiologic agent of Strangles, one of the most prevalent diseases of horses [1]. This strict-host equine pathogen is widespread around the world [2]. Nonetheless, the Icelandic herd is the only considered free of SEE because it is geographically isolated and is under a a law that has been in force for more than 1000 years prohibiting the importation of horses into the country [3]. This makes Iceland horses the gold standard for evaluations of serological tests for strangles diagnosis.

Within the suit of virulence factors expressed by SEE, several are highly immunogenic and targets for the development of vaccines and immunoassays. The SeM, for instance, is a constitutively expressed 58 kDa protein anchored in the SEE outer membrane that accumulates important functions for the virulence of the microorganism, such as the ability to bind fibrinogen and equine IgGs [4, 5]. Considered the most immunogenic of the SEE proteins, SeM

is present in all commercially available serological tests [6]. Although its antigenicity and immunogenicity characteristics make it a great target in serological tests, cross-reactivities are described with the M-like protein (SzM) present on the surface of *Streptococcus equi* subsp. *zooepidemicus* [7]. In addition, the SeM ability to bind IgGs must be considered when using it as an antigen in diagnostics, because even when the SeM is produced recombinantly, its characteristics of binding equine IgGs are preserved [5]. Along these same lines, the use of whole SEE cells as an antigen for serological tests is not recommended. Although there are commercial ELISAS available in the North American and European market [1] they are not found in Brazilian market. This leaves the serological diagnosis of SEE as well as the monitoring of herd immunity dependent on development of experimental tests. The aim of this study was to evaluate the use of a simple methodology in the extraction of surface antigens from SEE and its application in the development of an *in-house* iELISA.

2 Material and methods

2.1 SEE sodium deoxycholate antigen extraction

The protocol described by Ghoete et al. (2001) was used with some modifications for antigen extraction. First, the ATCC 39506 strain of *S. equi* subesp. *equi* (SEE) were cultivated on 5% blood agar plates overnight at 37 °C; then, the bacteria were harvested from plates and resuspended in 1.5 mL of Todd Hewit broth (THB) which was used to inoculate 1 L of THB broth (initial OD_{600} adjusted at 0.15). The bacteria culture was kept at 37ºC under agitation (150 rpm) up to reaching an OD_{600} of 1.2. The culture was centrifuged (8000×g, 20 min) and the pellet was resuspended in 20 mL of phosphate buffered saline (PBS). Then, 50 mM NaCl, 10 mM Tris-HCl and 0.025% of sodium deoxycholate (DOC) was added to SEE bacterial suspension. The suspension was kept under shaking (150 rpm) for 30 minutes at 37 °C and then

another centrifugation process in the same conditions described for culture broth was carried out. The supernatant containing the ATCC 39506 protein extract (SEE-DOC) was collected, dialyzed (100 mL of PBS) and concentrated to a final volume of 10 mL using an Amicon® Ultra 30 kDa centrifugal filter (Merk Millipore, USA).

2.2 Serum samples

For the in house iELISA standardization, a total 51 serum samples were used. Amongst these samples, 19 were considered negative because they came from pre-suckle foals. The other 32 samples were from horses that have recovered from strangles (*n*=5) and from animals vaccinated with a bacterin (*n*=27).

2.3 Immunoblotting

The proteins contained in the SEE-DOC were separated by SDS-PAGE using 5% acrylamide stacking gel overlaid on 10% acrylamide resolving gel. After SDS-PAGE, the proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, USA) using a semi-dry method (Electrosystems, Brazil). The membrane was then blocked overnight at 4 ℃ dipped in PBST-SK 3%. Sera collected from convalescent horse, diluted 1:100 in PBST-SK 1% was incubated with the membrane for 1 h at 22◦C under shaking. Then, the membrane was washed three times with PBS-T, during 10 min each. A rabbit anti-horse IgG Peroxidase conjugated (1:1.000) was incubated with the membrane under the same conditions as indicated for the sera. Three washes were performed before the membrane was incubated with peroxidase substrate (*4-Cloro-1-Naphthol* + 0.06% H2O2) for 10 min and then washed again in distilled water to stop the reaction.

2.4 In house iELISA design

Three polystyrene microplates (Microlon®, Greiner Bio One, Germany and Maxisorp®, Polysorp®, Nunc, USA) were tested to evaluate which would present the best adsorption capacity for the SEE DOC extract. A total of 10 positive serum samples were tested in triplicates on three different plates. All three microplates model were coated with 5µg/well of SEE DOC diluted in carbonate buffer (pH 9.6), during 2 h at 37 ℃ and then kept overnight at 4 ℃. After de adsorption process, plate wells were washed three times with phosphate buffered saline 0.05% Tween (PBST, pH 7.4) and blocked with 5% Skim Milk (Oxoid, UK) diluted in PBST (PBST-SK, at 37◦C for2 h. The positive horse sera were diluted 1:100 in PBST-SK 1% and 100 µL was added per well and incubated during 1 h at 37 ℃. After three PBST washing, peroxidase conjugated rabbit anti-horse IgG (Sigma-Aldrich, USA) diluted 1:5.000 in PBST-SK 1% was added and the plates were incubated as indicated above. Three more washes were performed prior to addition of substrate (3,3, 5,5'-tetrametilbenzidina + 0,06% H_2O_2). The plates were then incubated in the dark at room temperature for 10 min and the reaction was stopped by adding 50 µL of 3 N HCl. The optical density (OD) reading was performed at 450 nm using a Synergy HI plate reader (Bio-Tek®, USA).

Antigen concentration for coating was determined by titration in which twofold antigen dilution was added (100 µl/well) to the plates and evaluated against the 1:100 diluted positive sera. The optimal antigen concentration was defined as the lowest concentration that caused no significant changes in the OD obtained with the positive horse sera.

Ten positive and negative serum samples were used to determine the ideal dilution to be used in the iELISA. Serum samples were diluted 1:100, 1:200, 1:300 and 1:400 in PBST-SK 1% and 100µl of each dilution was added in duplicate to the sensitized plate wells (Maxisorp®, Nunc, USA). The optimal dilution was defined taken into consideration the mean absorption and the serum dilution [9].

2.5 Statistical analysis

The Kolmogorov-Smirnov test was used to determine the normal distribution of the data. The results were analyzed by Kruskal-Wallis or One Way Anova followed by Tukey or Sidak's post-test according to the data. Significant differences were considered when $p < 0.05$. All the statistics were performed using the GraphPad Prism software (GraphPad, USA).

3 Results

3.1 Antigen

At the end of the antigen extraction process using 0.025% Sodium Deoxycholate, the yield was 0.986 mg/mL of proteins solubilized in 10 mL final volume. Two main characteristics in the SEE-DOC were immediately evaluated. Firstly, the ability of SeM to bind IgG (non-immune reactions) through a Dot-Blot assay (not show), and the result confirmed that SEE-DOC does not present non-immune reactions with the Fc region of IgGs. Second, whether after extraction, the proteins present in the SEE-DOC maintained their antigenic characteristics. Moreover, as indicated by Western Blot, the serum of a Strangles convalescent animal was able to recognize different proteins present in the deoxycholate extract (Figure 1). To illustrate the inconvenience of using whole cells of SEE to sensitize plates in iELISA assays, a comparative graph of the results obtained by testing the same sera against SEE-DOC antigen and against whole cells of SEE ATCC strain was plotted (Figure 2).

3.2 Microplates, optimal antigen concentration and primary serum dilution

The MaxiSorp® and the Microlon® polystyrene microplates showed no differences in the adsorption capacity of SEE-DOC. However, both MaxiSorp® ($p=0.0016$) and the Microlon®

(p=0.0037) were more effective in SEE-DOC adsorption when compared with PolySorp (Figure 3).

The ideal concentration of SEE-DOC was determined by twofold serial dilution of the antigen adsorbed in a Maxisorp® microplate. The optimal quantity was 1.2 μg/well; at this antigen concentration the OD readings obtained from the positive control samples were similar to that obtained at the highest antigen concentration (5 μg/well) without significant differences (p=0.09). Then, for the remaining assays, the plates were coated with 1.2 μg/well of SEE-DOC 200 (Figure 4)..

The optimal primary sera dilution to be used in the iELISA was determined considering the positive/negative (P/N) OD relation. Both positive and negative horse serum samples diluted 1:100 had significantly higher OD values (p=0.003 and p=0.01 respectively) than when diluted 1:200 (Figure 5). From this result, the dilution of 1:100 was adopted for evaluating serum samples.

3.3 Specificity and sensitivity of iELISA

Nineteen serum from negative horses and 32 sera from positive horses were tested, under the optimized conditions described above, to determine values of cut-off, specificity and sensitivity. The ROC curve analysis of the iELISA data produced paired estimates of relative sensitivity and relative specificity at different cut-off values. A cut-off of OD ≥ 0.250 was recommended (Figure 6). At this cut-off value, the relative sensitivity and specificity estimates were 100% (95% confidence interval = 87.23% to 100%) and 95.9% (95% confidence interval = 71.32% to 99.85%), respectively. The Likelihood ratio at this cut-off was 17. The ROC curve (Fig 4) had an AUC value of 1.000 (95% confidence interval = 1.000 to 1.000), which indicated a high level of accuracy (Figure 7).

4 Discussion

Serological tests for the detection of antibodies against SEE are useful tools for strangles diagnosis [6]. In addition, they are essential for evaluating the potency and duration of vaccine-induced immunity, as well as for positioning vaccines in a sanitary program against strangles. In Brazil, unlike North America and Europe [1], there are no commercially available serological tests and, as such, we rely on diagnostic tools developed in-house. In the case of *Streptococcus equi* subsp. *Equi*, there are important obstacles for serological diagnoses: one of them is the cross-reactivity *with S. equi* subsp. z*ooepidemicus* [10]; another relates to the SeM, a constitutively protein expressed on the surface of SEE that has the ability to bind equine IgGs through the FC region (non-immune interactions) [5].

ELISA tests are the most frequently used for serological assays most likely due to easy execution and reproducibility of results, in addition to the ability to generate quantitative results (titer) of the induced antibody immune response. However, the antigens used to make these ELISAs present high variability. All commercials available uses recombinant SeM [1] proteins, or portions of them [7], in the antigen composition; the same happens with the experimental standardized ELISAs [10, 11]. However, the production of recombinant proteins is time-consuming and requires specific materials and expertise.

The outcome of this study is the standardization of an in house iELISA to detect antibodies against *S. equi* subsp. *equi*, using a method of extracting antigens from the bacterial surface that retains the antigenic characteristics of the extracted proteins but eliminates the non-immunological links between SeM and equine IgGs. Western blot using sera from a convalescent horse demonstrated the preserved antigenicity after extraction protocol of de SEE-DOC (Figure 1). The use of sodium deoxycholate for extracting surface exposed proteins was previously described for the production of immunogenic and antigenic vaccine antigens [8, 12]; however, as far as we know this is the first time this method has been used for an antigen used

in an iELISA. SeM (58 kDa) [11] is at least partially present in the SEE-DOC, indicated by the blot (Figure 1).

In order to assess the ability of SeM to bind IgGs through non-immune binding, the same sera used for the standardization of the iELISA with SEE-DOC were tested with plates sensitized with 15 Ug of whole cells from the ATCC 39506 strain (Figure 2). The results observed here corroborate those demonstrated by Meehan et al. (2001), which describe in detail the ability of SeM to bind IgGs from horses and other species by the Fc region, even when this protein was expressed and purified from *E. coli* in its native form. However, our results differ regarding the ability of SeM to bind rabbit IgGs [5]. When the secondary antibody (Rabbit anti-horse IgG) used here was tested on intact SEE cells without previous incubation with equine sera, recognition and consequent labeling was observed in dot-blot and ELISA assays (not show); this nonspecific labeling is not seen when using SEE-DOC, which does not lead to cross-reactivity between the secondary antibody and SEE. Interestingly, another study that standardizes the use of SeM as an antigen in an iELISA assay does not report these non-immune bindings after the expression and purification of native form rSeM [11]

Evaluation of the adsorption capacity of 3 different polystyrene microplates showed no differences between MaxiSorp® and Microlon® 600. Although not drastic, a decreased adsorption capacity of SEE-DOC compared to MaxiSorp® ($p=0.001$) and Microlon® 600 ($p=0.003$) was evidenced in PolySorp® microplates (Figure 3). Differences found here are most likely related to the hydrophobic and/or hydrophilic properties of the antigens (Pandolfi et al., 2017). Choosing the wrong plate can compromise the success of the assay [9, 13] strengthening the importance of testing the capability of different plates in binding antigens.

The optimal concentration of antigen used to sensitize MaxiSorp® plates was 1.2 µg/well, considered the lowest amount of antigen that did not significantly reduce the initial OD obtained with 5 µg/well (Figure 4). Even though this amount of antigen is apparently high,

compared to other works with the same objective [10, 11] it must be considered that in this type of extraction the target antigen is mixed with a suit of bacterial antigen. Despite being present and adding up at the time of measurement, may have different attachment capacity to the plate.

All 32 serum from vaccinated/exposed horses tested positive with an average OD of 1.648. Serum from 19 pre-suckle foals gave an average OD of 0.140. Using ROC curve analysis of the iELISA the cutoff was set at 0.250; at this set point, considering the horse population that was evaluated, the sensitivity of the assay was 100% and the specificity of 95,9%. Equine populations with very similar health status were used to standardize two other ELISA assays for the detection of antibodies against SEE: both used high sera dilution (1:2000) and an rSeM as antigen, and the cut-off values obtained were similar to this study 0.240 [10], and 0.374 [11], respectively.

By using serum from pre-suckle foals as negative samples, we may be neglecting the occurrence due to possible cross-reactivities between antibodies induced by infections caused by *S. equi* subsp. *zooepidemicus* (SEZ) [7], a commensal microorganism of horses, with worldwide distribution [2]). Pre-incubating sera with heat-killed *S. zooepidemicus* can remove, at least partially, cross-reactive antibodies to SzM prior to the detection of SeM-specific antibody responses [10]. Although the ability to discriminate between SzM cross reactive antibody has not been tested here, probably the fact that it is an antigen extracted from the surface of SEE, and apparently contains SeM, these cross-reactivities affect in some way the specificity of this iELISA. The adsorption process described above associated with a more conservative cut-off can help to differentiate specific and cross- reactions.

To date there is only one available test capable of separating antibodies against SEE from antibodies against SEZ, with high sensitivity (93.3%) and specificity (99.3%). This is a double iELISA, which uses the N-terminal recombinant protein fragments of SEQ2190 (antigen A) and SeM (antigen C) tested separately [7]. The specificity is guaranteed by the sampling of

139 serum from Icelandic horses, a population geographically isolated for over 1000 years. Consequently, Icelandic horses remained free from most common contagious diseases including strangles [3, 7].

5 Conclusion

The surface antigen extraction method with 0.025% Sodium Deoxycholate proved to be a simple and efficient method of producing antigens for antibody detection assays against SEE, for two main reasons: i) it eliminates non-immune reactions between SeM and equine IgGs; ii) antigenicity is preserved after the extraction process. More tests are needed, mainly regarding cross-reactivities with SEZ, but apparently, it has practical results similar to assays that use recombinant M proteins as antigen.

Acknowledgements

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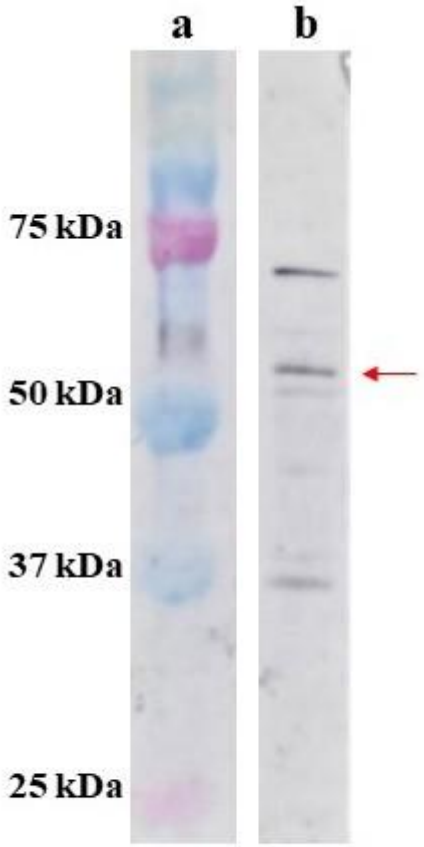

Figure 1. Western blot analysis of de SEE-DOC. After SDS-PAGE the antigen was transferred to nitrocellulose membrane. The antigenicity of SEE-DOC was evaluated by incubating the membrane (b) with a strangles convalescent equine sera. The presence of SeM in the extract is shown by the red arrow. a: molecular weight marker.

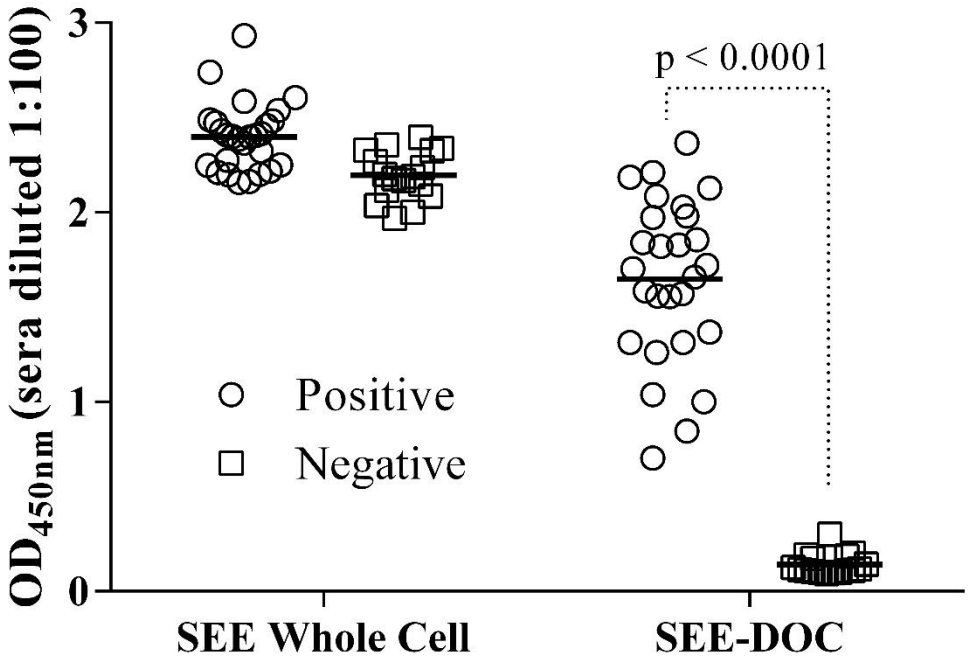

Figure 2. Comparison between SEE-DOC and whole SEE bacterial cell. All positive and negative sera were tested against whole SEE bacterial cell (15µg/well). The results were compared with those obtained with SEE-DOC iELISA.

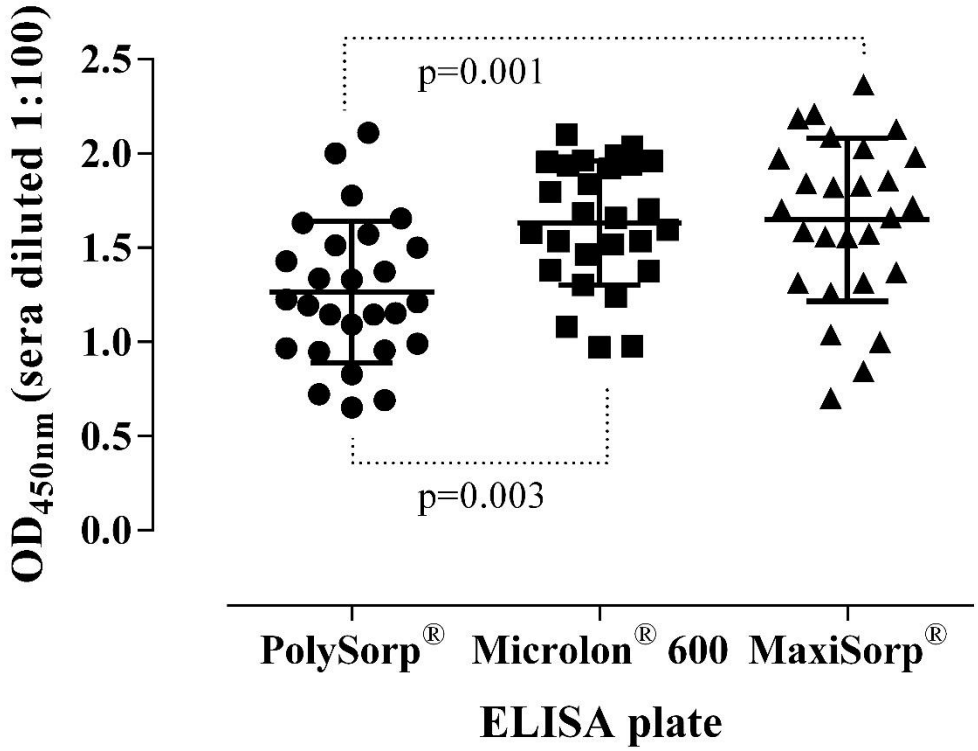

Figure 3. Performance of ELISA Maxisorp® Polysorp® and Microlon®600 microplates in the in house ELISA assay. Plates were sensitized with SEE-DOC (5µg/well) and reacted with 10 positive horse serum**.** Significant differences ($p<0.05$) are described in the graphic, with p values.

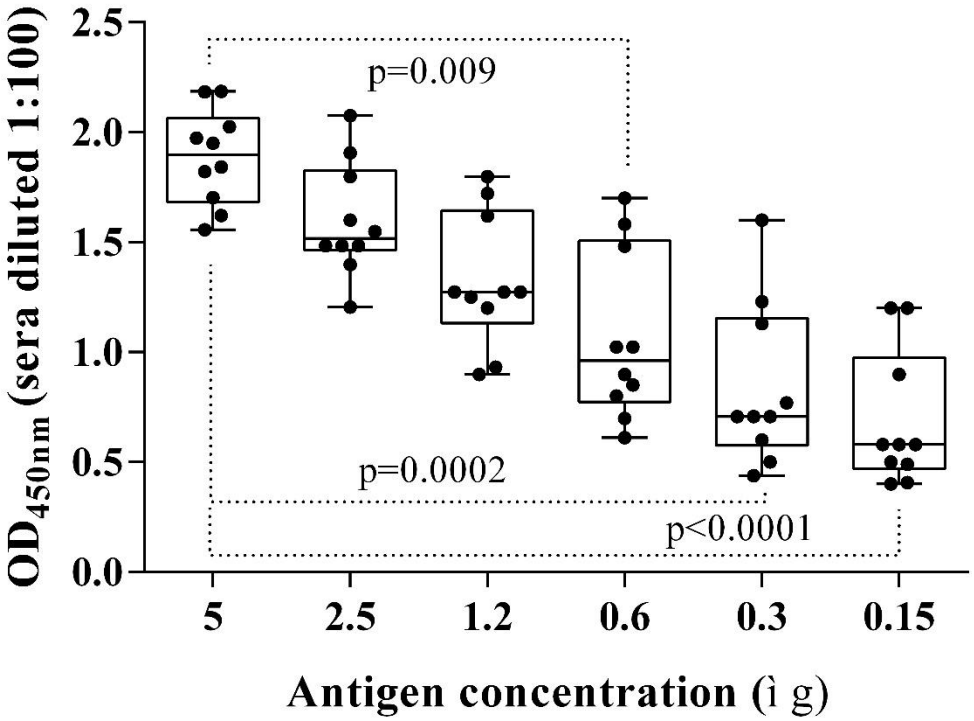

Figure 4. Optimal antigen concentration for the in house iELISA. SEE-DOC was twofold serially diluted before used to coat Maxisorp® microplate. Serum samples, previously determined to be positive (n = 32) were evaluated. The data is represented as the mean ± SEM of the OD of each antigen dilution. The optimal antigen concentration (1.2 µg/well) was defined as the lowest concentration that caused no significant changes in the OD.

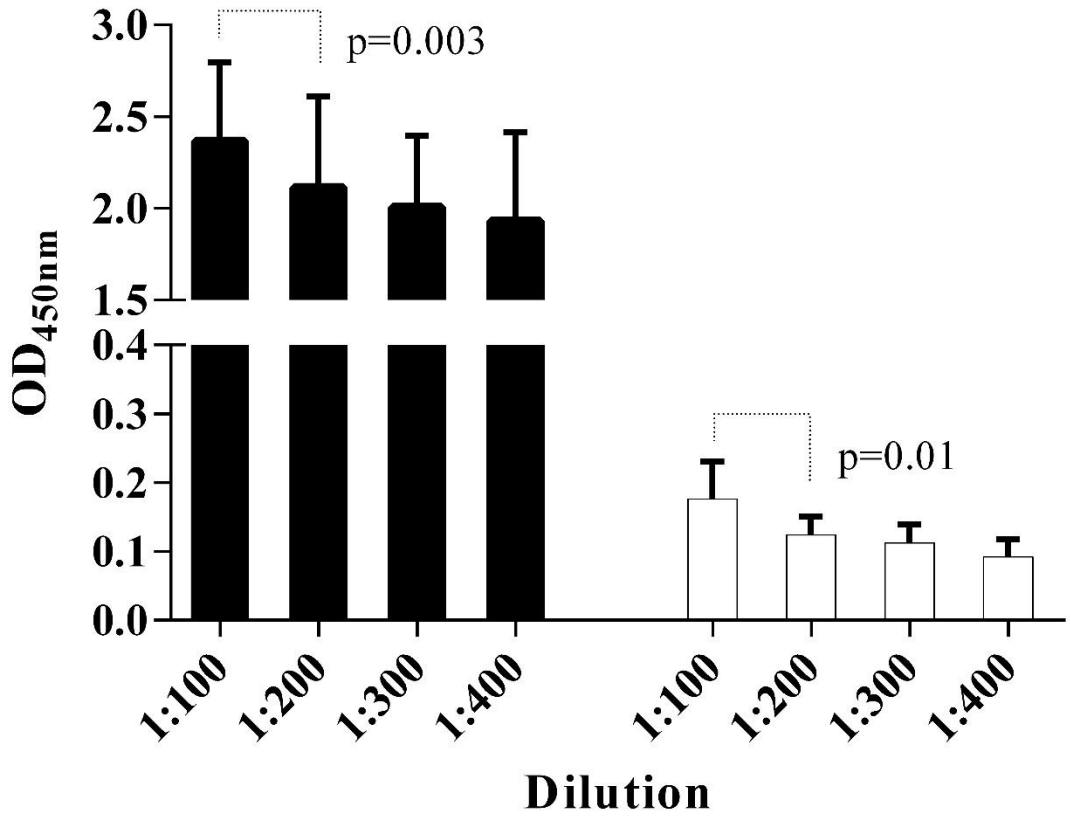

Figure 5. Determination of the primary serum dilution. Serum samples known to be positive (n = 10) or negative (n = 10) were diluted as indicated in the figure and tested for their ability to recognize SEE-DOC adsorbed onto Maxisorp® ELISA microplates. The p values indicates significant difference ($p<0.05$) between lowest serum dilution with to the immediately higher dilution within positive or negative samples.

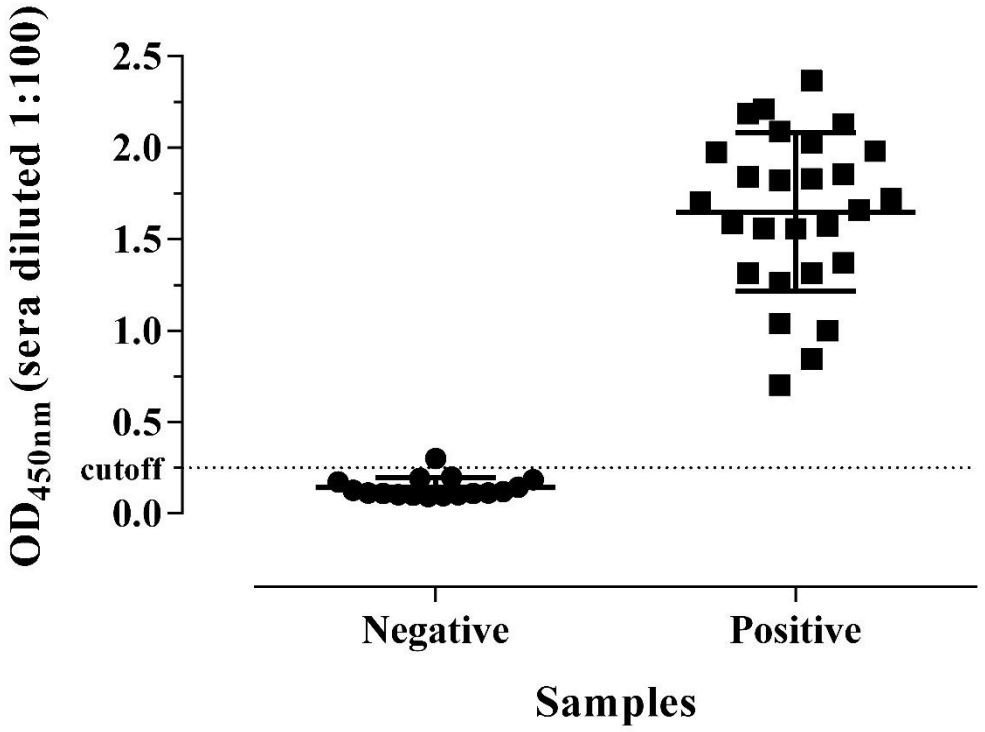

Figure 6. Positive and negative serum samples used to set de cutoff. The *in-house* ELISA was performed using evaluated optimal concentration of antigen (1.2 µg/well) and ideal serum dilution (1:100), with 51 serum samples. At OD_{450}, 0.250 cutoff value, the sensitivity and specificity were 100% (95% confidence interval = 87.23% to 100%) and 95.9% (95% confidence interval = 71.32% to 99.85%), respectively. The cutoff value is represented by the dashed line.

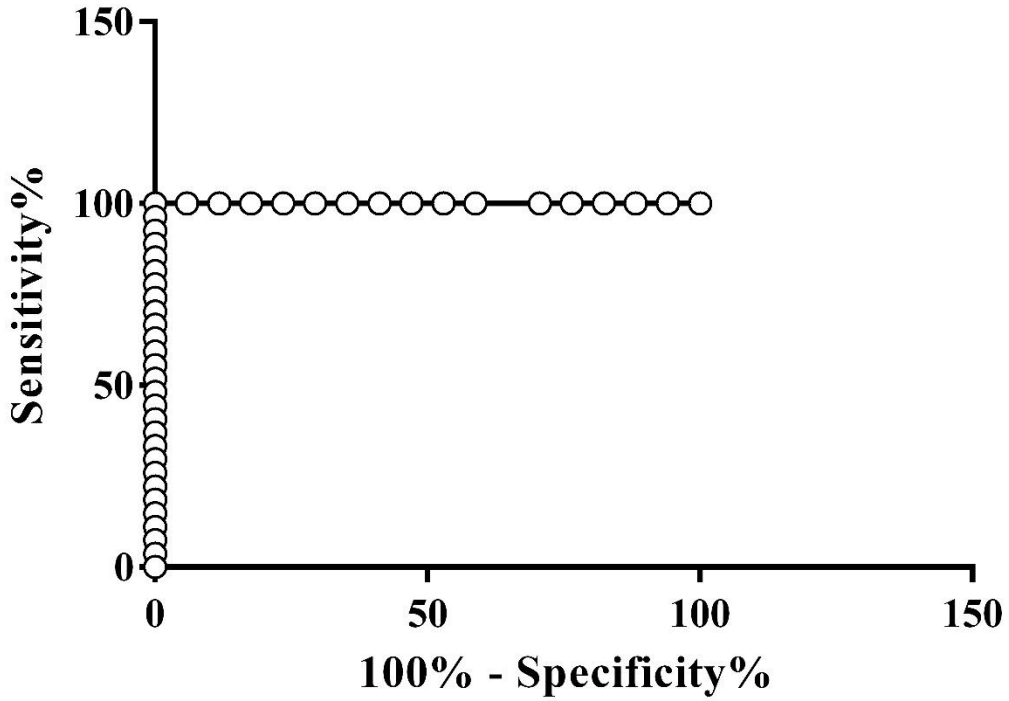

Figure 7. Receiver Operating Characteristic (ROC) analysis. The ROC curve was generated using the results obtained by analyzing 19 negative pre-suckle foals serum samples and 32 positive horses serum samples by the *in-house* iELISA. The area under the ROC curve was of 1.000.

4 MANUSCRITO 2

Vaccine formulation containing inactivated *S. equi* subsp. *equi* in whole culture media is highly immunogenic and protective in Swiss mice model

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Abstract

Vaccination plays a key role in preventing and controlling strangles outbreaks due to the ability to protect our horses both inside and outside the farm fence. They are harmless, and have marked efficiency when the strain involved in the outbreak has a similar antigenic profile to those present in the vaccine. The objective of this study is to evaluate the impact of the processes used in the vaccine antigen production, on the immunogenic and protective properties of bacterins. Four experimental vaccine formulations based on SEE inactivated whole cells, were evaluated. Four groups of 6 Swiss mice received one of the formulations, a control group of six animals, which received PBS. All groups were immunized twice with 14 days interval, and two weeks after the second immunization the mice were challenged with 10^6 SEE VE by the intranasal route. Blood samples to assess the titers and kinetics of IgG production were collected during the immunization protocol. After the experimental challenge, clinical signs, weight loss, survivor and colonization of the nasal cavity of mice were monitored. At the end of experiment, 83.3% of mice vaccinated with SEE VE based formulations survived the challenge. Protection in the groups immunized with SEE ATCC-based vaccines varied according to the antigen preparation. With the exception of the SEE ATCC PA, the others formulation resulted in significantly ($p<0.05$) superior protection compared to unvaccinated group. Mice vaccinated with the raw antigens (SEE VE and SEE ATCC strains) presented the lowest rate of colonization. IN terms of weight loss prevention, vaccines based on SEE VE strain was superior ($p<0.05$) compared to the unvaccinated group from day 4 post-infection. Likewise, the performance of these vaccines was statistically superior ($p<0.05$) in comparison to the vaccine based on SEE ATCC PA at 7 dpi. Independently to the SEE strain, vaccines formulated with RA induced biologically higher IgG titers compared to vaccines containing PA. Of the 4 vaccines developed, the formulation based on SEE VE RA strain was significantly more

immunogenic and induced a GMT of 224.The composition of SEE bacterin impacts in the immunogenic and protective properties of the vaccine formulation.

1. Introduction

Streptococcus equi subspecies *equi* (SEE) is the etiologic agent of Strangles, a highly contagious disease with worldwide distribution [1], that affect the upper respiratory tract of equines. After entering through the nasal and/or oral cavities, SEE reaches the tonsils and then it is drained via lymphatic circulation to the lymph nodes of the head and the neck region [2]. The infection is characterized by acute fever followed by pharyngitis, nasal discharge and abscess formation in the draining lymph nodes [3] which might rupture and release the purulent content. In some horses, with the pus drainage, the bacteria reach the guttural pouch causing empyema and the formation of chondroids, structures where SEE remains viable indefinitely [4]. Additionally, asymptomatic horses infected by well-adapted SEE strain can intermittently spread the bacteria for long periods [5]. Thus, the presence of SEE carriers is a matter of major concern to owners and veterinarians and makes the control and eradication of strangles an unmet goal [4, 6]; nonetheless, it is driving the efforts for developing vaccines with the potential to induce functional antibodies capable to prevent the SEE colonization and the clinical manifestation of the strangles.

Considering that convalescent animals develop long-lasting protective immunity, researchers are seeking to develop vaccines based on attenuated SEE strains (Pinnacle® - Zoetis and Equilis Strep E® - MSD) that can mimic the infection process and elicit the same immune response observed during the natural infection. The immune response induced by these vaccines are considered satisfactory but outbreaks of clinical disease in vaccinated young animals have been observed [7, 8]. In these cases, fail of protection might be related to antigenic differences between the vaccinal and disease-causing strain. Furthermore, the use of live attenuated

vaccines in immunosuppressed adult horses is not recommended, highlighting the need of efficacious inactivated vaccines to prevent SEE-associated disease.

In Brazil there are two inactivated, bacterin-based licensed vaccines aimed to prevent infection by SEE. The clinical efficacy of these vaccines has not yet been well demonstrated and because of negative field experience following their use, local veterinarians and horse owners are rather using autogenous vaccines to prevent strangles outbreaks (Dr. Castagna de Vargas, personal observation). In the short term, autogenous vaccines represent a good alternative but the development of a licensed vaccine with a broad-spectrum of protection is in great demand and urgently needed.

Currently, the efficacy of new vaccines against SEE is immunologically and clinically evaluated in horses or ponies [9]. The high cost of these animals along with the difficulties in finding animals serologically negative to SEE, the requirement of appropriate physical space and high cost of maintenance hurdles the development of new vaccines. Nonetheless, it instigates the search for alternative animal models to perform vaccines evaluation during the pre-clinical stage of a vaccine development [10,11].

In this scenario, this study presents a feasible strategy for preparing a new and promising vaccine against SEE. Using Swiss mice model, we found that antigen preparation and its availability in the vaccine formulation directly impact in the SEE-specific IgG response and clinical protection after an experimental challenge using a virulent field strain of SEE.

2. Materials and methods

2.1 Strains

The reference strain of *S. equi* subsp. *equi* (ATCC 39506) and a field strain named SEE VE were used here. The SEE VE strain was isolated from an outbreak of the strangles that affected 70% of the horses in a farm in the north of Rio Grande do Sul (RS) state, Brazil. The

bacterium was recovered from the submandibular lymph node of an adult horse with clinical signs of adenitis. To characterize the field strain we evaluated phenotypic characteristics, morphology, Gram staining and sugar fermentation (Lactose, Threhalose, Sorbitol and Ribose) by biochemical assay [12, 13]. Additionally, the differentiation of SEE and *Streptococcus equi* subsp. *zooepidemicus* was performed by multiplex PCR [14].

2.2 Bioethics and biosecurity committee approval

The procedures with animals were performed following the guidelines of the National Council for the Control of Animal Experimentation. The study protocol was approved by the Ethics Committee on the Use of Animals at the University of Passo Fundo (nº 047/2019).

2.3 Experimental reproduction of SEE-associated disease in Swiss mice model

To evaluate the protective capacity of the experimental vaccine we firstly standardized the SEE infection model in Swiss mice. For this purpose, 12 male Swiss mice were weighed (35.6 g ± 3.2) and randomly divided into three groups containing 4 animals each. Each group was housed in separate cages with water and feed *ad libitum* and a 12/12-hour dark/light cycle. After one week of acclimation, the mice were anesthetized with isoflurane and infected by the intranasal route with two concentrations of SEE VE (2×10^6 and 2×10^7 bacteria adjusted to a 10 µL volume in PBS). The mice were clinically evaluated (apathy, prostration and respiratory signs) and daily weighed during 5 days-post infection (dpi) [11].

2.4 Development of vaccine formulations

Four experimental vaccine formulations based on SEE inactivated whole cells (bacterins) were prepared; two containing the ATCC 39506 strain and two containing the SEE VE strain. For this purpose, the bacteria were initially cultivated on 5% blood agar plates (37

℃ for 12 to 16 hours); then, the bacteria were collected and suspended in 1 mL of Tryptic Soy Broth (TSB) which was used to inoculate 150 mL of TSB broth (initial OD_{600} adjusted at 0.1). The bacteria culture was kept at 37ºC under agitation (150 rpm) up to reaching an OD_{600} of 0.7 - 0.8 (~ 1×10^8 CFU/mL). At this moment the pH was adjusted to 7.4 and the whole culture was inactivated by adding formaldehyde (0.5% v/v) and incubation at 37ºC with agitation for 12 hours. From the inactivated cultures, two types of antigens were prepared, named: (a) raw antigen (RA) and (b) processed antigen (PA). The RA consisted of the TSB broth containing the whole mass of SEE (solid phase, soluble molecules and medium residues) without any additional steps after inactivation. The PA, in contrast, was obtained by complete removal of the TSB medium performed by three cycles of washing the bacterial pellet with 50 mL of 1X PBS. The formulation of the vaccines was performed in a class II biosafety hood. Briefly, a total of 20 mL of PA and/or RA containing 5×10^8 cells/mL, determined by flow cytometry [15], was added to a 100 mL beaker and kept under agitation (200 rpm) for 10 min. Then, 5% (v/v) of aluminum hydroxide (Omega Produtos Químicos, Brazil) was added and incubated under the same speed stirrer for 30 minutes at room temperature (22ºC). Then, the formulations were stocked in 50 mL sterile vials and kept at 4ºC until use. The sterility of each vaccine formulated was evaluated by inoculating 1 mL of each formulation in 9 mL of TSB broth and incubated at 37℃ for 14 days to assess bacteria growth.

2.5 Immunization and experimental challenge

The protective capacity of the vaccines was conducted in a Swiss mice model. A total of thirty mice 6 to 7 weeks of aged were weighed and randomly divided into 5 homogeneous groups (n = 6 each group) and then housed as described in section *2.3*. Two groups were immunized with bacterin from the SEE VE strain: G1 with RA and G2 with PA. And two groups were immunized with bacterin from the SEE ATCC strain: G3 with RA and G4 with

PA. Mice from group G5 were inoculated with PBS containing 5% (v/v) of Aluminum Hydroxide. All mice were inoculated twice (day 0 and day 14) by subcutaneous injection of 0.2 ml of each formulation. On day 28 all animals were anesthetized (as described above) and challenged intranasally with the SEE VE strain (2×10^6 bacteria/mice). Pre-challenging blood samples were collected from all mice on the inoculation day (D0), at booster inoculation (D14) and challenging day (D28).

2.6 Protective capacity of vaccines in relation to animal weight and respiratory bacterial load

Clinical evaluation was assessed during 10 days post challenge (dpc) following the protocol described previously [11] with some modifications. In addition to daily weight loss (%) and the colonization of the nasal cavity, the presence of dyspnea and prostration were also evaluated. The animals were weighed (43.1 g ± 1.5) at the pre-challenge moment (D0) and then daily during 10 d.p.c. To ealuate the colonization of the nasal cavity, mice were manually restrained and nostrils imprinted directly on the surface of a 5% sheep blood agar plate. Then, 50 µL of sterile 1X PBS were added on the contact spot and spreading of the content was carried out using sterile glass beads to allow counting of the number of colony forming units (CFU). The growth of bacteria on plates was rated on a scale of 0 to 3, where 0 corresponded to growth of <5 CFU, 1 corresponded to growth of 5-100 CFU, 2 corresponded to growth of 101 - 300 CFU, and 3 corresponded to uncountable CFU.

2.7 Evaluation of the humoral immune response induced by the formulations

The production of serum antibody on mice was evaluated by an indirect *in-house* ELISA. 96-well plates (MaxiSorp®, Nunc, USA) were coated with 1 µg/well of an outer membrane proteins antigenic extract obtained from the SEE ATCC strain by 0.025% sodium deoxycholate treatment [16, 17]. The kinetic of the antibody response (qualitative analysis) was carried out

using sera (diluted 1:50) collected on D0, D14 and D28. The absolute IgG titers were determined in serum samples collected at D28 and, for this purpose, sera were serially diluted (1:50 to 1:6,400) in PBST (PBS+0.05% Tween 20) containing 1% skim milk (SM). Then 100 µL of diluted sera were added to each well and the plates were incubated for 1 hour at 37°C. Afterward, the wells were washed 3 times with 250 µL PBST and then 100 µL of peroxidase-conjugated anti-mouse IgG secondary antibody (diluted 1:5,000 in PBST + SM) was added and incubated for 1 hour at 37 °C. Finally, 3 washes were performed and 100 µl of 3,3,5,5'-tetramethylbenzidine (Sigma Aldrich, USA) + 0.06% H_2O_2 (Sigma Aldrich, USA) were added and the plates incubated for 10 minutes in the dark. The enzymatic reaction was stopped by adding 50 µL of 3M HCl. Color intensity was detected at 450 nm with Synergy HI plate reader (Bio-Tek, USA). The IgG titer was defined as the reciprocal of the highest dilution with an absorbance value greater than twice the mean absorbance of sera from the unvaccinated group. The Geometric Mean of Titre (GMT) was calculated with the Graph Pad PrismTM software (Graph Pas Software, San Diego, California, USA).

2.8 Necropsy and isolation

All animals that died as a consequence of infection or that were euthanized at the end of the experiment were necropsied. Heart blood and fragments of submandibular lymph node and lung and were collected for isolation of SEE. Samples were plated on 5% sheep blood agar and incubated at 37 °C for 24 hours.

2.9 Statistical analysis

Data were analyzed using the Graph Pad Prism™ program (Graph Pas Software, San Diego, California, USA). Level and absolute titers of SEE-specific IgG was analyzed using the two-way ANOVA test, followed by Sidak's multiple comparison test. The same strategy was

used to compare weight loss after the experimental challenge. Survival analysis was conducted with the Kaplan-Meier test. P values are indicated in the figure legends.

3 Results

3.1 Standardization of Swiss mice model to reproduce SEE-associated disease

All infected mice showed significant ($p<0.0001$) and progressive daily weight loss (%) compared to uninfected animals over the 5-dpi period (Figure 1A); in both groups the accumulation of weight loss was approximately 30% at 5 dpi. Conversely, the uninfected group had an average weight gain of 9% and 10% in the same period. In the clinical evaluation, signs of apathy and respiratory distress were observed in the first 24 hours, which were more pronounced in animals infected with 2×10^7 cells. At 3 and 4 dpi severe respiratory distress, ocular secretion and clinical signs compatible with conjunctivitis were observed in animals infected with 2×10^7 and 2×10^6 SEE. Only one animal from the group infected with 2×10^7 died at 5 dpi as a consequence of the experimental infection. Overall the two selected doses of infection were capable to trigger the clinical disease and avoid an acute systemic chock, which is not desirable to evaluate the protection performance of an experimental vaccine against a respiratory disease.

Regarding the kinetics of nasal cavity colonization, animals infected with 2×10^6 SEE had significantly ($p<0.01$) more bacteria in the nasal cavity compared to those infected with 2×10^7 SEE at 2 dpi (Figure 1B); in both infected groups, the highest number of CFU was recovered at 3 dpi. As expected, SEE was not isolated from any uninfected animals.

At necropsy, submandibular lymph nodes were augmented in both infected groups and two animals from the group challenged with 2×10^7, and one from the group challenged with 2×10^6, presented lymph node abscessation (Figure 2).

All mice, except one from the group infected with the lower dose, were positive for the isolation of SEE in at least one of the samples analyzed (lung fragment, submandibular lymph node and whole blood).

3.2 Vaccines-induced antibody response

The kinetics of IgGs response induced by the four different vaccine formulations were evaluated during the pre-challenging period (D0, D14 and D28) and the absolute IgGs titers was determined at D28. All vaccines induced SEE-specific IgGs on mice and the highest levels of antibodies were detected at D28 (Figure 3A), except on mice vaccinated with SE ATCC PA.

Before the challenge (D28), vaccines formulated with RA (independently to the SEE strain) induced biologically higher IgG titers compared to vaccines containing PA (Figure 3B). Of the 4 vaccines developed, the formulation based on SEE VE RA strain was significantly more immunogenic and induced a GMT of 224.

3.3 Clinical protection induced by experimental vaccines

To evaluate the capacity of the experimental vaccines to inducing clinical protection, we challenged all mice 14 days after revaccination (D28). The mice were infected by the intranasal route with 2×10^6 SEE VE bacteria. During the clinical evaluation period, non-vaccinated animals showed apathy (6/6), dyspnea and ocular secretion (5/6) (Table 2). The severity of clinical signs started to increase from 3rd dpi and due to the severity of infection, 100% of unvaccinated animals died (4/6) or were sacrificed (2/6) to mitigate unnecessary suffering (Figure 4A). On the other hand, mice immunized with vaccines based on SEE VE antigen presented slight clinical signs, characterized mainly by apathy during the first 5 dpi; group (G1) immunized with SEE VE RA had less affected animals than group (G2) immunized with SEE VE PA. Dyspnea was observed in one animal from both groups on day 7 post-infection (Table

1). Animals immunized with SEE ATCC strain presented on extra day of apathy in comparison to those immunized with SEE VE-derived antigen; and mice immunized with the vaccine based on SEE ATCC PA (G4) developed lung disease similar to unvaccinated mice (Table 1). Interesting, no mice that received the vaccine based on SEE VE RA developed coryza; on the contrary, in the other groups we detected at least one mouse with ocular secretion (Table 1).

As clinical signs can affect the average of daily weight gain, we assessed the mice body weight daily post-infection. The two vaccines based on SEE VE strain significantly prevented ($p<0.05$) weight loss in comparison to the unvaccinated mice from day 4 post-infection onwards. Likewise, the performance of these vaccines was statistically superior ($p<0.05$) in comparison to the vaccine based on SEE ATCC PA at 7 dpi. The trend of weight loss observed in the group that received the vaccine based on SEE ATCC RA was biologically inferior that those observed in the groups that received vaccines based on SEE VE between days 4 to 6 post-infection (Figure 4B).

In terms of survival, 83.3% of mice vaccinated with SEE VE RA or PA were full protected against the challenge based on SEE VE strain (strain homologous to the vaccine antigen) (Figure 4A). The protection observed in the groups immunized SEE ATCC-based vaccines varied according to the antigen preparation.

In the group immunized with SEE ATCC RA 66.6% of the mice were protected against challenging and only 33.3% of mice immunized with SEE ATCC PA survived to challenging by SEE VE strain (heterologous related-vaccine strain) (Figure 4A). Hence, with the exception of the SEE ATCC PA vaccine, the others formulation resulted in significantly ($p<0.05$) superior protection against SEE infection compared to unvaccinated group. Finally, mice vaccinated with the raw antigens (SEE VE and SEE ATCC strains) presented the lowest rate of colonization between all evaluated mice (Figure 3C and 3D). These results highlight the promising use of this type of antigen to formulate vaccines to horses.

3.4 Pathologic analysis and SEE recovery from systemic sites

No evidence of lymphadenopathy was observed in mice from G1 and G2 groups. In contrast, 33.3% of mice immunized with SEE ATCC RA (G3) had lymphadenopathy characterized by suppurative abscess in the submandibular lymph node, right side. The rate of lymphadenopathy was even higher (66,6%) in mice vaccinated with the SEE ATCC PA-based vaccine (G4), that displayed bilateral abscesses in the submandibular lymph nodes, and one animal had lung abscess. Within unvaccinated mice, 83.3% of developed severe lymphadenopathy and 33.3% had lung abscesses.

In addition, the lowest rate of bacterial recovery was found on mice immunized with vaccines containing SEE VE (Table 2). With one exception (mice #4 that died from infection), no bacteria could be recovered from mice immunized with SEE VE RA (G 1) and a similar trend was observed in mice from groups G2 and G3. In contrast, SEE was isolated from the lung and heart blood from 66.6% of mice immunized with SEE ATCC PA and a similar trend was observed within mice from the unvaccinated group.

4 Discussion

The data obtained in our study indicate that RA prepared from SEE strains cultures induces protection against homologous and heterologous challenge in a mice model. Here, protection was measured as improved survival rate, absence or reduced colonization of lymph nodes by the challenging bacteria and improved anti-SEE antibody production to the immunizing antigen. Protection conferred by immunizing with RA was superior compared to PA most likely because SEE soluble antigens present on culturing media, but not on bacterin, might have a key role in inducing protective immunity. Also, our data supports current evidences that live-attenuated [7] and multiple recombinant antigen-based vaccines [9] induce better protection in that a broader range of antigens are presented to the vaccinated animals, inducing antibodies to

a wider range of key epitopes on antigens that might have central role on SEE pathogenicity. Furthermore, the mice we used developed clinical signs that resemble those observed in horses with strangles strengthening the use of mice as a model to evaluate different aspects of SEE infection.

Several strategies have been used to developing a broad-spectrum vaccine against equine adenitis. Natural infections are known to inducing a potent and long-lasting antibody response [18]; therefore, the use of vaccines containing live attenuated SEE strains has been the subject of research by several groups [7, 19, 20]. However, the live attenuated TW 928 strain (Equilis StrepE vaccine) has been reported to induce a short-lasting immune response of 3 months approximately [21]. In addition, some vaccinated animals had local reactions at the submucosal or intramuscular administration site [20] and some had clinical manifestations of strangles [8]. The Pinacle strain used in another commercial live attenuated vaccine has restrictions regarding its use in foals and might cause clinical sign of adenitis in vaccinated animals; also, it was isolated from sick, but previously vaccinated animals [22]. These data demonstrate that commercially available, live-attenuated SEE vaccines are not completely safe to all vaccinated animals and the immune response provided is incomplete even after SEE replication on the vaccinated animal.

Currently, the focus on the development of vaccines against strangles has turned to formulations using recombinant proteins as antigens [23, 24, 25]. In this segment, two formulations should be highlighted: Septavac, that contains 7 recombinantly produced SEE surface proteins, which confers 85% protection in ponies challenged by the intranasal route [24]; and Strangvac 4, which contains 8 SEE outer membrane proteins fused to 3 antigens [25]. These vaccines are safe and succeeded in reducing clinical signs and inflammatory parameters in animals but fail to a large extend in preventing lymph nodes colonization by SEE, resulting in abscesses formation in 14 out of 16 vaccinated ponies [9]. And, additionally, they induce and

humoral response that allows differentiating infected from vaccinated animals which is a hallmark for eradication program. However, although promising, both vaccines are still not available for field use and their cost-effectiveness for scale production should be carefully evaluated.

Considering that the most effective and promising vaccines against strangles require a combination of several recombinant protein antigens [24, 25], it suggest that an effective immune response against SEE infections should be driven against multiple epitopes. Indeed, here we found that bacterin formulated with RA associated to modulation of the immune response by the appropriate adjuvant are capable to prevent clinical sings of equine adenitis. Vaccines formulated with inactivated whole microorganisms are widely and long-time used to control bacterial and viral infections worldwide, besides being the only vaccine model available in Brazil against strangles. However, studies addressing the improvement of SEE immunization using bacterin are scarce. In this context, our main goal was to evaluate 4 different bacterin formulations focusing on the SEE cell mass processing to find out the best approach to have antigenic and immunogenic antigens to compose a bacterin-based vaccine.

Additionally, we aimed to standardize an infection experimental model using Swiss mice as feasible animals to be used in other studies like this. Because horses are not often used in the early stages of vaccine development, for several reasons, such as high cost of the animals and maintenance, and human empathy, the use of laboratory animals, when biological compatible, represent an excellent alternative. In this sense, we firstly evaluate the use of the Swiss mice as an experimental challenge model to reproduce SEE infection as well respiratory disease. Based on an infection protocol [10] and monitoring of clinical and microbiological and pathological parameters, we verified the biological compatibility of Swiss mice to reproduce SEE clinical manifestation as seen in naturally infected horses. Thus, we demonstrated that SEE VE was able to adhere and multiply efficiently in mice upper respiratory tract, resulting in

clinical respiratory signs, abscess formation and increase in submandibular lymph nodes size, and progressive weight loss (Figure 1A and B). Furthermore, vaccinated mice were protected by vaccination with SEE RA indicating the induction of protective immunity. Although our study is not a pioneer in the validation of murine models, our results suggest that the heterogeneous Swiss mouse can be used to replace Balb/c, C57BL/6, Laca and NMRI models [10, 26, 23, 27, 28] making this kind of experimental more accessible to groups working in this field. Our clinical findings after the Swiss mice infection via the intranasal route were similar to the SEE pathogenesis in horses [29], with an increase in submandibular lymph nodes and colonization of the upper respiratory tract, as well as the results described by Chanter et al. (1995) during Balb/c mice validation as experimental model for SEE infection.

The present study also evaluated the protective capacity of four experimental vaccines, formulated using the same adjuvant (Al(OH)3) to enhance two distinct antigen presentations of SEE ATCC and SEE VE strains. All formulations induced the production of serum IgGs, being the O.D. significantly different after the second immunization, except in the processed SEE ATCC formulation, demonstrating that the 14-day interval was sufficient to induce a good response to the boosting immunization. Regardless of the strain used, our findings demonstrate the superiority of RA compared to PA to inducing high level of IgGs, regardless the strain (Figure 4). Observing the quantitative results obtained through the titration of IgGs induced by different formulations, it is evident that the strains have differences in immunogenicity. When comparing the different strains used in the same antigen formulation, we observed that the SEE VE strain induced higher serum IgG titers. According to Galán and Timoney (1988) and Timoney et al. (2010) failures in cross-protection would not be related to antigenic divergence among SEE strains due their very homogeneous antigenic pattern [30, 31]. This information, along with our results added to the differences found in the quantification of IgGs between SEE ATCC and SEE VE may suggest that there are differences in the amount of surface antigen

expression resulting in greater capacity to stimulate humoral-based responses. Furthermore, we highlight the importance of characterizing the immunogenic capacity of SEE strains before its use in a vaccine formulation, even more in cases that there are two or more strains composing the antigenic unit.

Although both SEE VE formulations resulted in similar survival rates against homologous challenge, parameters as weight loss, nasal cavity colonization, and SEE recovery from systemic sites, showed a superior performance of animals immunized with SEE VE RA. Conversely, mice immunized with SEE ATCC showed different survival rates, being 66% (4/6) and 33% (2/6) for SEE ATCC RA and PA, respectively, against heterologous challenge. As observed with SEE VE, SEE ATCC RA induced superior protection compared to PA based on the analysis of weight loss, nasal cavity colonization and SEE recovery from systemic sites. In summary, the results found here demonstrate that there is a deficiency of cross protection between the two strains used in the formulations, and that RA are apparently superior to modulate a protective response against heterologous challenge. These findings corroborate, at least in part, with the field reports of commercial bacterin unsatisfactory coverage against SEE infection. In contrast, our results diverge from those reported by Bazeley (1942), where an antiserum produced against a single SEE strain protected mice against 32 SEE strains during an experimental challenge [32].

Apparently, the best way to prevent SEE infection is through antibodies capable of neutralizing the agent before it reaches the tonsils [33], as a result of robust immune response at the upper respiratory tract (URT) mucosa. Although we did not assess the mucosal immune response induced by the formulations, the results of nasal cavity colonization demonstrated that vaccine formulation containing RA were able to induce a response at URT mucosal level. The idea that vaccines produced with whole inactivated microorganisms and administered by the intramuscular route do not induce immune response at the mucosal level is common, but might

be contested. In fact, there is a study evaluating two commercial vaccines to prevent proliferative enteropathy in swine caused by *Lawsonia intracellularis* (a pathogen that also affects horses), that demonstrates superior protection at the intestinal mucosa level, associated with reduced *L. intracellularis* fecal excretion in pigs intramuscularly vaccinated with a bacterin compared to pigs orally vaccinated with a live attenuated strain [34]. Here, a possible explanation for the lower rate of nasal cavity colonization in animals immunized with formulation containing RA may be based on the evaluation of serum IgG titers induced, which were higher in mice vaccinated with these formulations. Thus, a larger amount of IgGs may have been translocated to the mucosa of the URT, reducing SEE replication at this site.

5 Conclusion

The composition of SEE bacterin impacts in the immunogenic and protective properties of the vaccine formulation. We demonstrate that SEE vaccines immunogenicity and antigenicity varies according to the strain used. In this work, the SEE VE strain proved to be superior in terms of immunogenicity, indicating that it is a good choice to compose a vaccine formulation against strangles. Finally, Swiss mouse is an excellent experimental model for SEE infection and, consequently, to evaluate vaccines against equine strangles.

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Table 1. Clinical signs observed during *Streptococcus equi* subesp *equi* infection in mice vaccinated and unvaccinated.

Groups	Days post-infection Number of affected mice/totals of alive animals									
	1	2	3	4	5	6	7	8	9	10
	Apathy									
G1 - SEE VE raw ag	6/6	4/6	1/6	1/5	1/5	0/5	0/5	0/5	0/5	0/5
G2 - SEE VE processed ag	6/6	3/6	3/6	3/5	3/5	0/5	0/5	0/5	0/5	0/5
G3 - SEE ATCC raw ag	6/6	3/6	2/6	2/6	2/6	2/6	0/4	0/4	0/4	0/4
G4 - SEE ATCC processed ag	6/6	1/6	1/5	3/5	3/5	3/4	0/2	0/2	0/2	0/2
G5 - Unvaccinated	6/6	4/6	4/6	6/6	4/6	1/1	0/0	0/0	0/0	0/0
	Dyspnea									
G1 - SEE VE raw ag	0/6	0/6	1/6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
G2 - SEE VE processed ag	0/6	0/6	1/6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
G3 - SEE ATCC raw ag	0/6	0/6	0/6	0/6	1/6	2/6	0/4	0/4	0/4	0/4
G4 - SEE ATCC processed ag	0/6	0/6	1/5	3/5	3/5	3/4	0/2	0/2	0/2	0/2
G5 - Unvaccinated	0/6	0/6	4/6	4/6	4/6	1/1	0/0	0/0	0/0	0/0
	Ocular secretion - coryza									
G1 - SEE VE raw ag	0/6	0/6	0/6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
G2 - SEE VE processed ag	0/6	0/6	0/6	1/5	1/5	0/5	0/5	0/5	0/5	0/5
G3 - SEE ATCC raw ag	0/6	0/6	0/6	0/6	1/6	1/6	0/4	0/4	2/4	2/4
G4 - SEE ATCC processed ag	0/6	0/6	0/6	1/5	1/5	1/4	0/2	0/2	0/2	0/2
G5 - Unvaccinated	0/6	0/6	3/6	5/6	5/6	1/1	0/0	0/0	0/0	0/0

Table 2. Isolation of *Streptococcus equi* subsp. *Equi* from systemic sites of vaccinated, unvaccinated, and experimentally challenged animals.

Vaccine formulation	Animal identification	Anatomical sites		
		Submandibular lymph node	Lung	Heart
G1 - SEE VE raw antigen	**1**	-	-	-
	2	-	-	-
	3	-	-	-
	4	+	+	+
	5	-	-	-
	6	-	-	-
G2 - SEE VE processed antigen	**1**	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	+
	6	+	-	-
G3 - SEE ATCC raw antigen	**1**	+	+	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
G4 - SEE ATCC processed antigen	**1**	-	-	-
	2	+	+	+
	3	-	+	+
	4	-	+	+
	5	+	+	+
	6	-	-	-
G5 - Unvaccinated	**1**	+	+	+
	2	+	+	+
	3	-	+	+
	4	+	-	-
	5	-	+	-
	6	+	+	+

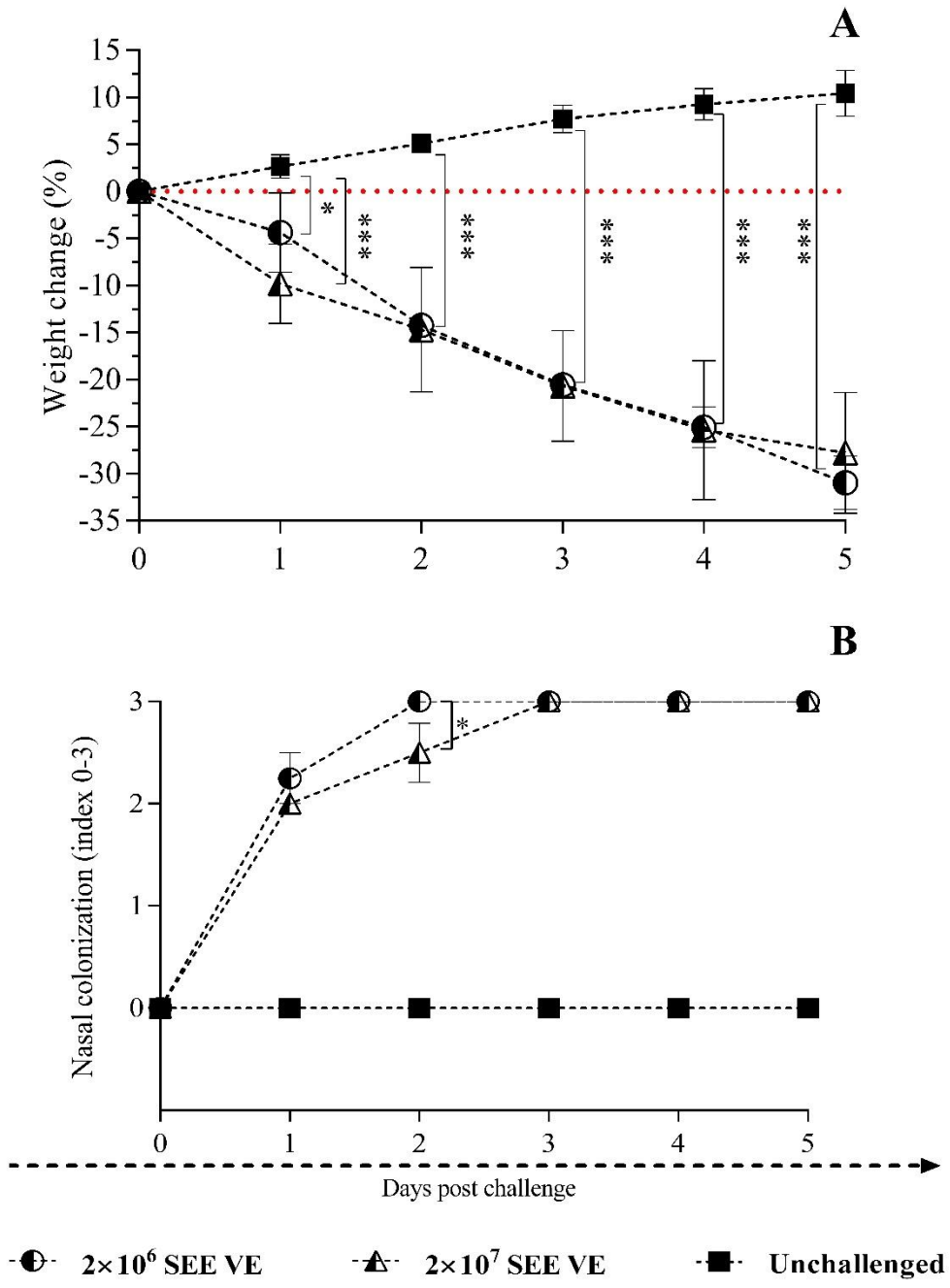

Figure 1. A. Cumulative body weight gain and loss produced by SEE infection in Swiss mice model. A total of 12 mice was randomically distributed into three experimental groups of 4 animals each. Two groups were intranasally infected with 2×10^6 and 2×10^7 SEE VE cells and one group (uninfected) received 1X PBS by the same route. Body weight was measured daily. Weight loss or gain is represented as a percentage (mean ± standard deviation). Statistical differences between infected and uninfected groups are represented with asterisks (*p<0.01 and **** p<0.0001). **B. SEE kinetics of upper respiratory colonization.** Respiratory colonization was determined by directly detection of SEE through isolation and quantification. Bacterial load was classified according to the following scale: 0 = < 5 CFU; 1=5-100 UFC; 2=100-300 UFC; 3=Countless UFCs. The data is represented with the mean ± standard deviation. Statistical difference is represented with an asterisk (*p<0.01).

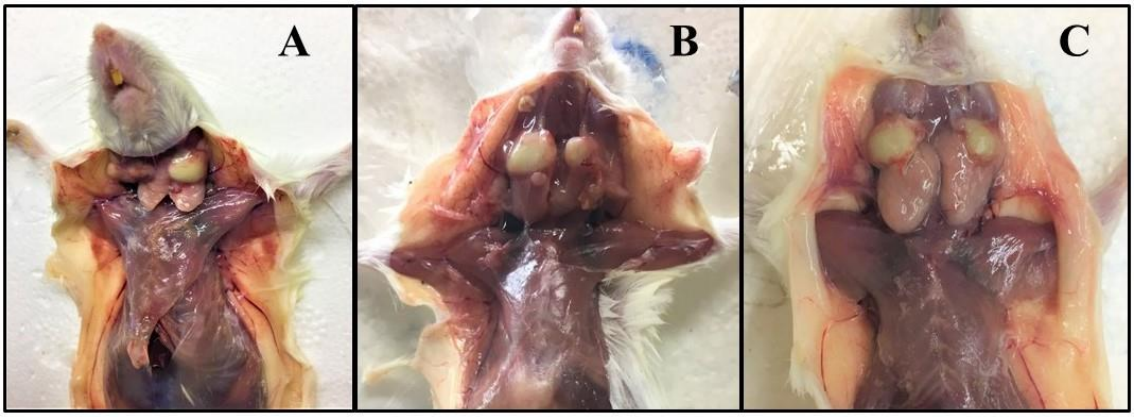

Figure 2. Lymphadenopathy induced by SEE infection in Swiss mice model. Submandibular lymph nodes with abscess formation after SEE VE intranasal challenge is indicated by red arrow. A. Mice from group challenged with 2×10^6 SEE VE cells. B and C. Mice from group challenged with 2×10^7 SEE VE cells.

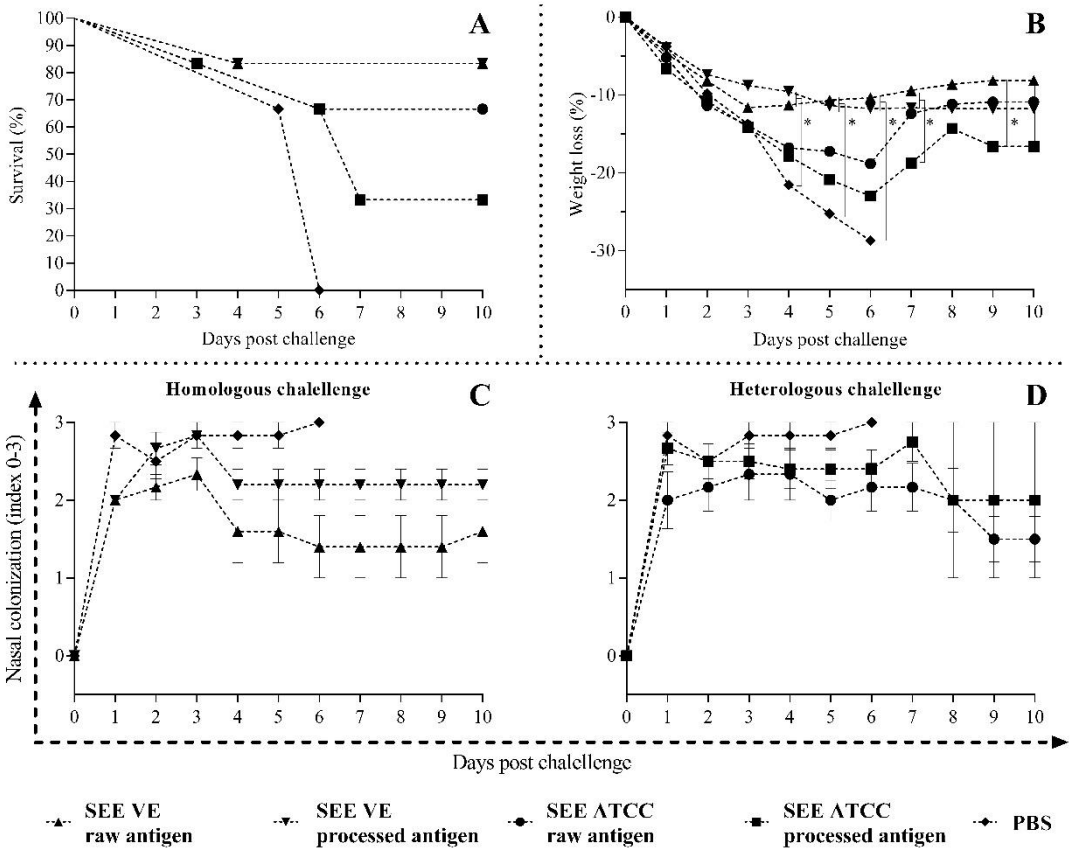

Figure 3. Fourteen days after revaccination, all animals were challenged intranasally with 2×10^6 cells of the SEE VE strain. The body weight was measured daily. **A. Clinical survival rate of mice immunized and challenged.** After the challenge (D28) the animals were kept under observation for 10 days. **B. Changes in body weight measured during 10 days after challenge.** Weight loss is represented as percentage (mean ± SD). Statistical differences between groups are represented with asterisks (*$p<0.01$). **C. Nasal colonization index of mice vaccinated with SEE VE antigens. D. Nasal colonization index of mice vaccinated with SEE ATCC antigens.** The nasal colonization is presented as an index (mean ± SEM), that ranges from 0 to 3. Where 0 corresponded to growth of <5 CFUs, 1 corresponded to growth of 5-100 CFUs, 2 corresponded to growth of 101 - 300 CFUs, and 3 corresponded to uncountable CFUs.

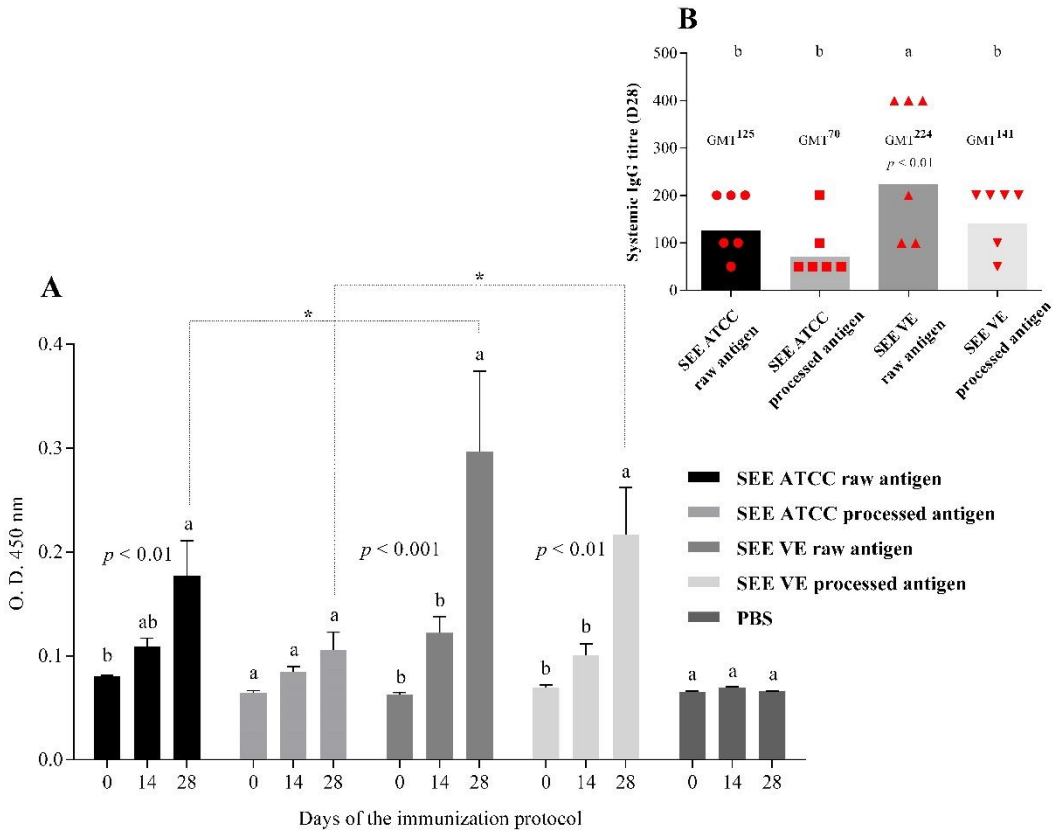

Figure 4. Serological response induced by experimental vaccines based on SEE ATCC and SEE VE strains. **A.** The IgG production kinetics represents the mean ± standard deviation of the absorbance of serums diluted 1:100 and collected at moments D0 (pre-vaccination), D14 (revaccination) and D28 (experimental challenge). **B.** The IgG titer was determined at D28 and is represented as the geometric mean vaccine titer (GMT). Different letters indicate statistical differences between moments within each group. On the other hand, differences between vaccines are represented with asterisks (*p<0.01).

5 DISCUSSÃO

Os resultados apresentados nesta tese se referem a ferramentas diagnósticas e imunoprofiláticas capazes de auxiliar no controle do garrotilho.

No primeiro estudo um método de extração de antígenos de superfície de células inteiras de SEE foi otimizado para produção de antígeno o qual foi então utilizado posteriormente para a padronização de um ELISA indireto.

Considerando a população avaliada, o ELISA indireto padronizado apresentou resultados satisfatórios, com 100% de sensibilidade e 95,9% de especificidade, utilizando um *cutoff* de 0,250 na OD_{450}. Desta maneira, todos os 32 soros oriundos de animais com histórico prévio de adenite, ou vacinação com bacterinas de SEE, foram apontados como positivos. E, ao contrário, foram considerados negativos todos os 19 soros de potros privados de colostro, os quais apresentaram valores de OD_{450} abaixo de 0.250.

Para aumentarmos os níveis de confiança nos resultados gerado pelo iELISA são necessárias avaliações com maior número de amostras de soro; e, dentre estas amostras, é de suma importância dispormos de soros de animais convencionais que possuam um histórico de infecções, ou vacinações, estritamente conhecidos, como os utilizados para a padronização do iELISA. O fato de haver reatividade cruzada entre SEE e SEZ, somado a ampla disseminação do SEE nos rebanhos brasileiros e internacionais, fica praticamente impossível termos dentro da amostragem animais adultos convencionais, livres destes patógenos. Isso pode ser solucionado de duas maneiras: a) pela importação de testes de ELISA em especial o descrito por (ROBINSON et al., 2013) ou, b) pelo uso de soros provenientes de equinos que vivem na Islândia (BJÖRNSDÓTTIR et al., 2017). Este último seria o padrão ouro para a validação de um ensaio de ELISA para detecção de anticorpos específicos contra SEE.

Outra metodologia a ser testada é a utilização de células inteiras de SEZ inativadas por calor (este protocolo deve ser padronizado) para adsorção dos soros de equinos, previamente à testagem contra SEE (DAVIDSON et al., 2008). No entanto para se utilizar a adsorção dos soros, as diluições dos anticorpos primários devem ser novamente testadas e calculadas novo *cutoff* pela avaliação através da curva ROC.

Ao avaliarmos dois métodos de processamento de cultivos de SEE empregados na produção de bacterinas, encontramos diferenças nas características de proteção e antigenicidade das formulações. Ambas apresentavam a mesma quantidade de antígeno (1×10^7/dose), quantificado em número absoluto de células por citometria de fluxo; também apresentavam a mesma concentração (5%) do adjuvante hidróxido de alumínio (AlOH). Ao final do

experimento, 83,3% dos camundongos vacinados com formulações baseadas em SEE VE sobreviveram ao desafio. A proteção nos grupos imunizados com vacinas à base de SEE ATCC variou de acordo com a preparação do antígeno, no qual a utilização de AB foi superior na proteção. Com exceção da SEE ATCC AP, as demais formulações resultaram em proteção superior ($p<0,05$) em relação ao grupo não vacinado. Camundongos vacinados com os AB de ambas as cepas apresentaram a menor taxa de colonização. Interessante, independentemente da cepa de SEE utilizada, as vacinas formuladas com antígenos brutos induziram títulos de IgG biologicamente mais altos em comparação com vacinas contendo PA. OS resultados demosntraram que a metodologia utilizada para o processamento da unidade antigênica de bacterinas de SEE altera a capacidade protetora e titulos de anticorpos em camundongos. Adicionamos aos resultados encontrados aqui outras possibilidade que, associadas, poderiam aumentar a capacidade protetora das bacterinas, como testes com adjuvantes e enriquecimento das bacterinas com subunidades imunogenicas de SEE, a exemplo do que foi testado por (ROSA et al., 2021)

Devido a sua utilização em diversas modalidades esportivas, os equinos são transportados com uma frequência incomparável a outros animais de produção: somente no ano de 2016 foram registradas 150.000 negociações envolvendo equinos entre países da união europeia (MITCHELL et al., 2021). Com isso, o controle de enfermidades infecciosas nos equinos fica comprometida; nestes casos, a presença de imunidade protetora contra um determinado patógeno pode evitar grandes prejuízos.

Na profilaxia vacinal, além da necessidade da utilização formulações vacinais com capacidade de indução de respostas imunes protetoras, são necessárias ferramentas que possibilitem a avaliação da potência e duração da imunidade induzida pela vacinação. Neste sentido, os estudos aqui apresentados são complementares, ressaltando que foi necessário o desenvolvimento de uma metodologia que apresentasse resultados seguros na avaliação sorológica anterior aos estudos de avaliação da composição antigênica das bacterinas. A campo, estas ferramentas são e devem ser utilizadas de forma a se complementarem, como antes mencionados. Ensaios que permitam avaliações sorológicas quantitativas, como é o caso dos iELISAs, são os maiores aliados no desenho de um programa de vacinação para a adenite equina e qualquer outra doença para a qual existe algum método de imunização. Contar com ensaios que possibilitem posicionar vacinas de bom desempenho, de maneira a diminuir a janela imunológica de potros e estimular a imunidade de animais adultos em momentos oportunos são extremamente necessárias no combate ao garrotilho.

6 CONCLUSÃO

Nesta tese foram desenvolvidos estudos com capacidade de impactar de maneira imediata no diagnóstico e profilaxia vacinal da adenite equina no Brasil, uma vez que ambos os estudos trazem soluções simplificadas para avaliação e incremento da imunidade frente a *Streptococcus equi* subespécie *equi n*os equinos.

Primeiramente, o ensaio de ELISA indireto padronizado e avaliado possui uma característica que facilita muito a produção deste tipo de ensaio para detecção de anticorpos anti-SEE no soro de equinos. Isso ocorre pelo fato do antígeno utilizado para sensibilização das placas ser um extrato de cultivo total e não uma proteína recombinante.

Por segundo, até o momento, as bacterinas são as únicas vacinas contra a adenite equina possíveis de utilização no Brasil. Aqui, demonstramos que as características de imunogenicidade e capacidade protetora destas formulações vacinais depende fundamentalmente na maneira como as proteínas são extraídas dos cultivos bacterianos. Além disso, as cepas utilizadas na formulação destas vacinas, principalmente em formulações polivalentes, devem obrigatoriamente ser previamente avaliadas quanto às suas características imunogênicas. Essas informações podem ser utilizadas de imediato nas plataformas já existentes de produção de bacterinas.

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