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Dissertação

Ensaio de imunoadsorção enzimática como diagnóstico de esporotricose felina

Débora Matilde de Almeida

Pelotas, 2023

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Banca examinadora:

Prof.ª Drª Márcia de Oliveira Nobre (Orientador)
Doutora em ciências veterinárias pela Universidade Federal do Rio Grande do Sul

Prof.ª Drª Renata Osório de Faria
Doutora em ciências veterinárias pela Universidade Federal do Rio Grande do Sul

Prof.ª Drª Natasha Rodrigues de Oliveira
Doutora em ciências pela Universidade Federal de Pelotas

Prof.ª Drª Fabiane Borelli Grecco
Doutora em ciências veterinárias pela Universidade Federal de Pelotas

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''Todas as graças da mente e do coração se escapam quando o propósito não é firme.''
William Shakespeare

Resumo

ALMEIDA, Débora Matilde de. **Ensaio de imunoadsorção enzimática como diagnóstico de esporotricose felina.** 2023. 79f. Dissertação (Mestrado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2023.

A esporotricose é uma doença infecciosa causada pelo fungo *Sporothrix spp*. e é considerada uma zoonose de grande importância médica e veterinária. Embora seja uma doença que pode afetar várias espécies animais e seres humanos, a infecção é mais prevalente em gatos, sendo considerada uma das principais causas de lesões cutâneas nesses animais. O diagnóstico da esporotricose é baseado em uma combinação de achados clínicos, histórico e exames laboratoriais. O método de referência para o diagnóstico é o isolamento do agente em cultura micológica, seguido da caracterização morfológica e conversão para forma leveduriforme. No entanto, esse método pode ser demorado e nem sempre é sensível o suficiente para detectar a infecção em estágios iniciais. Nesse contexto, os testes sorológicos, especialmente o Ensaio de imunoabsorção enzimática (ELISA), têm sido estudados como uma ferramenta promissora para auxiliar no diagnóstico, pois são rápidos, não invasivos e podem ser realizados em larga escala. Neste contexto, o objetivo deste estudo foi desenvolver um ELISA indireto para a esporotricose felina utilizando um antígeno recombinante. O artigo 1 aborda desenvolvimento de teste de ELISA utilizando a proteína quimérica rChiGp70_Eno de *S. brasiliensis* como antígeno. O teste mostrou excelente desempenho, a sensibilidade foi de 88% (IC 95%: 70%-96%) enquanto a especificidade foi de 100% (IC 95%: 85%-100%). O artigo 2 é uma revisão sistemática e meta-análise que teve como objetivo sumarizar a atual evidência encontrada na literatura científica sobre o ELISA para diagnóstico da esporotricose em humanos e gatos. A meta-análise agregou os dados de sete diferentes estudos desenvolvidos em um intervalo de 17 anos. O ELISA se mostrou outra vez como uma excelente ferramenta para auxiliar no diagnóstico da esporotricose, mostrando uma sensibilidade de 93% (IC 95%: 90%-95%) e especificidade de 91% (IC 95%: 89%-93%). O ELISA indireto é uma excelente ferramenta para auxiliar o diagnóstico da esporotricose, baseado em seu potencial de ser um teste mais rápido comparado ao método padrão, além do seu alto nível de sensibilidade e especificidade.

Palavras-chave: diagnóstico; gatos; ELISA; sorologia; proteína recombinante

Abstract

ALMEIDA, Débora Matilde de. **Enzyme-linked immunosorbent assay as diagnostic of feline sporotrichosis.** 2023. 79f. Dissertation (Master degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2023.

Sporotrichosis is an infectious disease caused by the fungus *Sporothrix spp.* and is considered a zoonosis of significant medical and veterinary importance. Although the disease can affect multiple animal species and humans, the infection is most prevalent in cats, where it is regarded as one of the leading causes of cutaneous lesions. The diagnosis of sporotrichosis is based on a combination of clinical findings, patient history, and laboratory tests. The reference method for diagnosis is the isolation of the causative agent through mycological culture, followed by morphological characterization and conversion to the yeast form. However, this method can be time-consuming and may not always possess sufficient sensitivity to detect the infection in its early stages. In this context, serological tests, particularly the Enzyme-Linked Immunosorbent Assay (ELISA), have been studied as a promising diagnostic tool due to their rapid, non-invasive nature and the potential for large-scale application. The objective of this study was to develop an indirect ELISA for feline sporotrichosis using a recombinant antigen. Article 1 discusses the development of an ELISA test using the chimeric protein rChiGp70_Eno from *S. brasiliensis* as the antigen. The test demonstrated excellent performance, with a sensitivity of 88% (95% CI: 70%-96%) and a specificity of 100% (95% CI: 85%-100%). Article 2 is a systematic review and meta-analysis aimed at summarizing the current evidence in the scientific literature regarding the use of ELISA for diagnosing sporotrichosis in humans and cats. The meta-analysis consolidated data from seven different studies conducted over a 17-year period. ELISA once again proved to be an excellent tool for aiding the diagnosis of sporotrichosis, demonstrating a sensitivity of 93% (95% CI: 90%-95%) and a specificity of 91% (95% CI: 89%-93%). The indirect ELISA is an excellent diagnostic tool for sporotrichosis, given its potential to serve as a faster alternative to the standard method, in addition to its high sensitivity and specificity.

Keywords: diagnosis; cats; ELISA; serology; recombinant protein

Lista de Figuras

Lista de Abreviaturas e Siglas

AUC	Area Under the Curve
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEEA	Comitê de Ética e Experimentação Animal
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
D.O	Densidade óptica
DAB	3,3'-diaminobenzidina
DOR	Diagnostic Odds Ratio
ELISA	Enzyme-Linked Immunosorbent Assay
FAPERGS	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
FelV	Feline Leukemia Virus
FIV	Feline Immunodeficiency Virus
FN	False Negative
FN	Falso Negativo
FP	False Positive
FP	Falso Positivo
GMS	Grocott-Gomori
H_2O_2	Peróxido de Oxigênio
HCl	Ácido Clorídrico
HE	Hematoxilina e Eosina
IC	Intervalo de Confiança
IgG	Imunoglobulina G
IPTG	Isopropil β-D-1-tiogalactopiranosida
LB	Luria Bertani
LILACS	Literatura Latino-americana e do Caribe em Ciências da Saúde
MeSH	Medical Subject Headings
$NiSO_4$	Sulfato de Níquel
NLR	Negative Likelihood Ratio

PAS	Periodic Acid–Schiff
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween 20
PCR	Polymerase Chain Reaction
PLR	Positive Likelihood Ratio
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QUADAS-2	Quality Assessment of Diagnostic Accuracy Studies 2
ROC	Receiver Operating Characteristic
SROC	Summary Receiver Operating Characteristic
TN	True Negative
TP	True Positive
VN	Verdadeiro Negativo
VP	Verdadeiro Positivo

Lista de Símbolos

<	Menor
>	Maior
°C	Grau Celsius
A	Ampere
g	Grama
h	Hora
kDa	Quilodáltons
Kg	Kilograma
Mg	Miligrama
M	Molar
mM	Milimolar
ng	Nanograma
nm	Nanômetro
pH	Potencial Hidrogênico
rpm	Rotações por Minuto
V	Voltz
µm	Micrômetro
µl	Microlitro

Sumário

1 Introdução

A esporotricose é uma doença zoonótica causada por fungos dimórficos do complexo *Sporothrix* spp. O *S. brasiliensis* é a espécie mais virulenta do clado clínico, e os felinos são a espécie mais acometida (GREMIÃO et al., 2020). Os animais se contaminam com o fungo inserido em habitat natural, pelo costume de arranhar cascas de árvore e contato com matéria orgânica, ou através de arranhadura e mordedura em agressões intraespécie (RAMÍREZ-SOTO et al., 2018).

O diagnóstico definitivo é estabelecido através do isolamento do agente etiológico por meio de cultura micológica e observação de leveduras na caracterização morfológica (DE MIRANDA et al., 2018). Outros métodos também podem ser utilizados como estratégias de acompanhamento ou pré-diagnóstico, como o exame citológico e exame histopatológico (GREMIÃO et al., 2020). No entanto, pelo crescimento do fungo ser lento na forma leveduriforme e a necessidade de que o laboratório seja certificado como nível 2 de biossegurança para o diagnóstico definitivo, pode ocorrer a demora no resultado e prejuízo no manejo e tratamento do paciente (SILVA et al., 2018).

Observadas as dificuldades encontradas na realização do diagnóstico convencional, outras alternativas estão sendo aplicadas. (FERNANDES et al., 2011). O uso do ensaio de imunoadsorção enzimática (ELISA) indireto como ferramenta diagnóstica se baseia na possibilidade de um teste mais rápido comparado ao método padrão, com alta sensibilidade e especificidade (ALVARADO et al., 2015). Em felinos, há apenas um estudo utilizando o *S. brasiliensis* na forma de exoantígeno (RODRIGUES et al., 2015b).

O uso de fragmentos imunogênicos para desenvolvimento de proteínas recombinantes para diagnóstico de doenças zoonóticas já tem sido explorado na medicina veterinária (FERNANDES et al., 2022). Por meio do estudo de componentes da parede celular do *Spororothrix* spp, glicoproteínas de 60-70 kDa (Gp70) e a proteína enolase de 47 kDa (SsEno) tem sido utilizadas como antígenos imunodominantes, porém a maioria dos estudos tem como enfoque a produção de vacinas (DE ALMEIDA et al., 2018; PORTUONDO et al., 2022). Desta forma, considerando a importância zoonótica da esporotricose, bem como os agravos do aumento de casos da doença no Brasil e no mundo, e a possibilidade de ampliar as

estratégias diagnósticas com o auxílio da biotecnologia o desenvolvimento de proteínas recombinantes, desenvolveu-se o presente trabalho.

2 Revisão da Literatura

2.1 Histórico

A esporotricose é uma micose de implantação causada pelo complexo *Sporothrix* spp., afetando a pele e o tecido subcutâneo (FISHER, 2001). A doença é transmitida pela inoculação traumática do agente pelo contato com solo e atividades de jardinagem ou por mordidas e arranhaduras de gatos contaminados (RAMÍREZ-SOTO et al., 2018). A partir da análise de conteúdo coletado de abcessos de lesões em braço e mão, o *Sporothrix schenckii* foi isolado pela primeira vez em 1898 por um estudante de medicina nos Estados Unidos (HEKTOEN; PERKINS, 1900). No Brasil, o primeiro caso de esporotricose foi relatado por em 1907, por infecção natural em ratos e humanos (LUTZ; SPLENDORE, 1907).

2.2 Epidemiologia

Com o avanço das pesquisas e da análise molecular, o pensamento que o *S. schenckii* era o único agente da doença foi sendo modificado (MARIMON et al., 2006). Por meio da avaliação filogenética da calmodulina, novas espécies foram propostas e atualmente o complexo *Sporothrix* spp. compreende 53 espécies divididas em dois tipos de clado: clínico e ambiental (DE BEER; DUONG; WINGFIELD, 2016; RODRIGUES et al., 2020). As espécies de interesse ambiental apresentam baixo potencial patogênico em hospedeiros de sangue quente e são referidas como *S. chilensis, S. humicola, S. mexicana* e *S. pallida*. Já a *S. phasma* também está inserida no clado ambiental, porém sem fator virulento em mamíferos (RODRIGUES et al., 2022). Geralmente são encontradas em materiais orgânicos como o solo, casca de árvore, restos de animais e madeira em estado de decomposição (RAMÍREZ-SOTO et al., 2018). As espécies do clado clínico são responsáveis pelos casos de infecção, sendo referidas como *S. schenckii sensu stricto, S. globosa, S. luriei* e *S. brasiliensis*, esta última sendo a mais virulenta em felinos (MARIMON et al., 2007; RODRIGUES; HAGEN; DE CAMARGO, 2022).

Por mais que seja uma micose encontrada em todo mundo, esta distribuição não ocorre de modo uniforme (CHAKRABARTI et al., 2015). Na Europa, os casos primários foram relatados na França (BEURMANN; GOUGEROT, 1910), no entanto,

em estudos recentes, cerca de 12 isolados de *Sporothrix* spp. foram relatados no continente europeu, cujo só na Alemanha foram encontradas seis cepas. Boa parte dos isolados se referiam as espécies do clado ambiental, como *S. cantabriensis, S. euskadiensis, S. mexicana, S. nebularis* e *S. pallida* (MORGADO et al., 2022). Na Ásia, a maioria dos diagnósticos são atribuídos ao *S. globosa*, com relato de casos no Japão, Malásia, China e na Índia (HAN; KANO, 2020; RUDRAMURTHY et al., 2020).

Na América Latina, há relatos de casos na Argentina, Colômbia, Peru, Venezuela e Brasil, este último, considerado o epicentro da esporotricose, principalmente atribuído ao estado do Rio de Janeiro onde a notificação da doença é obrigatória (BARRETO et al., 2021; ETCHECOPAZ et al., 2021; GALLO et al., 2022; GREMIÃO et al., 2020a; RAMÍREZ SOTO, 2015). Em estudos de revisão e epidemiologia, cerca de cinco mil casos humanos e cinco mil casos felinos foram reportados entre 1998 e 2020 (GREMIÃO et al., 2020a; RABELLO et al., 2022). Surpreendentemente, no último boletim epidemiológico do estado do Rio de Janeiro 2616 pacientes foram confirmados com a doença, de acordo com critérios clínico-epidemiológicos ou laboratoriais (SES, 2021). Independente se em humanos e animais, a maioria dos casos ocorrem por meio de mordedura e arranhadura por gatos e a infecção geralmente é ocasionada pelo *S. brasiliensis* (ALVAREZ; OLIVEIRA; PIRES, 2022).

2.3 Aspectos clínicos

A esporotricose já foi descrita em diversas espécies, como canídeos, murinos, equídeos, ruminantes; no entanto, o gato é o mais acometido por essa doença (BOECHAT et al., 2020; CROTHERS et al., 2009; DALIS et al., 2014). Nestes animais, a micose pode se manifestar de forma cutânea, linfática ou disseminada, cujo a última pode ser fatal (LLORET et al., 2013). As lesões são geralmente são encontradas na região cefálica, em plano nasal e orelhas, e na região de membros e posterior. Para melhor manejo, os gatos podem ter as lesões classificadas com L1, em que há lesão em apenas um local; L2, duas ou mais lesões cutâneas em diferentes locais; L3, lesões cutâneas em três ou mais locais, de forma não contínua (SCHUBACH et al., 2004). Do ponto de vista clinico-dermatológico, as lesões são nodulares de textura firme, podendo apresentar conteúdo exsudativo de coloração marrom e também

crostas (LLORET et al., 2013) (Figura 1). Em casos graves, lesões necróticas com envolvimento muscular e ósseo também podem estar presentes.

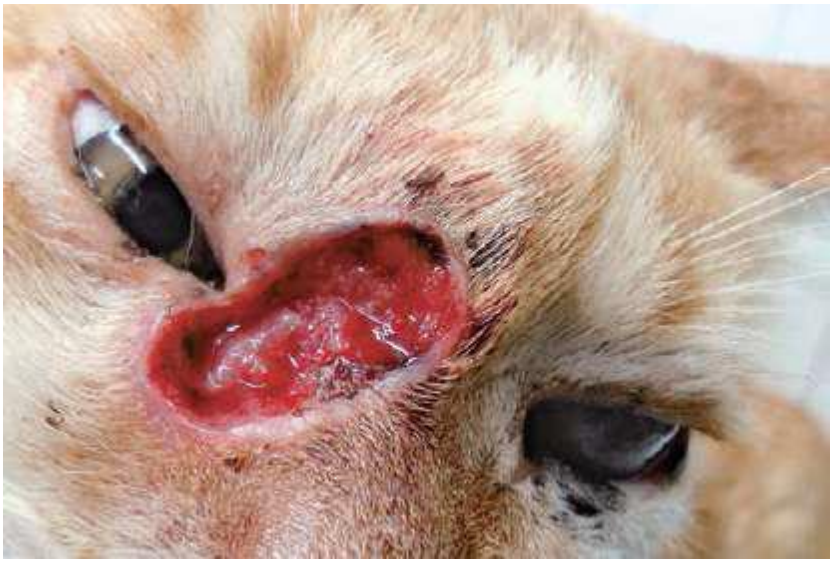

Figura 1 – Lesão ulcerativa na face de um felino acometido por esporotricose (JERICÓ, 2015).

Quando o acometimento é sistêmico, sinais respiratórios, anorexia, febre, hipotermia, desidratação e outros sinais de descompensação são relatados (WELSH, 2003). Os gatos ainda podem ser classificados de acordo com seu estado geral em que S1 refere ao estado geral bom, sem sinais extracutâneos; S2, estado geral regular, com sinais sistêmicos extracutâneos leves; S3, com estado geral delicado, com sinais extracutâneos e S4, estado geral crítico, com sinais extracutâneos intensos (MIRANDA et al., 2013).

2.4 Tratamento

A esporotricose felina é uma doença de difícil resolução, pois além do impacto das características e gravidades das lesões, existe um número limitado de medicamentos a serem utilizados, tem-se grandes relatos de falha terapêutica ou recrudescência bem como grande taxa de abandono do tratamento (GREMIÃO et al., 2015; LLORET et al., 2013; NASCIMENTO, 2019). O manejo terapêutico é iniciado de acordo com a avaliação completa do histórico do animal, correlacionando com os

sinais clínicos cutâneos e se tiver, sinais sistêmicos bem como a classificação das lesões (GREMIÃO et al., 2020b).

Para gatos acometidos com lesões únicas, o uso do itraconazol como monoterapia é indicada, utilizando por dia 100 mg se o animal tem mais de 3 Kg, 50 mg se peso maior que 1 Kg porém inferior a 3 Kg e, para animais com menos de 1 Kg, o uso de 25 mg/Kg é o recomendado (GREMIÃO et al., 2020b). Para casos refratários com o uso da monoterapia ou se o gato apresentar múltiplas lesões e/ou sinais respiratórios e/ou lesões em mucosa, o uso do itraconazol associado com iodeto de potássio 2,5-5 mg/Kg é preferível, principalmente se estes animais nunca foram medicados (REIS et al., 2016).

Para casos mais graves e residuais que ainda apresentem múltiplas lesões, o uso de anfotericina B, bem como tratamentos alternativos como termoterapia e crioterapia também podem ser avaliados (DE SOUZA et al., 2016; HONSE et al., 2010). A cura é estabelecida a partir do desaparecimento de todos os sinais clínicos, o que pode alongar o tempo de tratamento. Além disso, é preciso manter o tratamento por um mês após o desaparecimento dos sinais clínicos, principalmente caso o animal tenha inicialmente apresentado lesões na região nasal ou sinais respiratórios visto a possibilidade de recidivas (LLORET et al., 2013).

2.5 Diagnóstico

O diagnóstico da esporotricose felina é obtido pela correlação das informações sobre o histórico, sinais clínicos, contexto epidemiológico e exames laboratoriais coletados do paciente (BARROS; DE ALMEIDA PAES; SCHUBACH, 2011; GREMIÃO et al., 2020b). Todas essas informações são necessárias visto que os sinais clínicos são inespecíficos, por exemplo, as lesões cutâneas podem ser semelhantes a de criptococose, leishmaniose e outras doenças que causam dermatites (OROFINO-COSTA et al., 2017). Além disso, o diagnóstico da doença bem como a correta identificação do agente permite que estratégias de vigilância epidemiológica e promoção de políticas públicas de saúde sejam criadas e o conceito saúde única seja expandido (ROSSOW et al., 2020).

2.5.1 Exame micológico

O método de referência para diagnóstico de esporotricose é realizado por isolamento do *Sporothrix* spp. em cultura micológica, seguido da identificação por caracterização morfológica e conversão para a fase leveduriforme (KWON-CHUNG; BENNETT, 1992). As amostras podem ser obtidas por meio de exsudato, crostas, secreções e fragmentos de órgãos coletados por meio *swab, imprint*, aspirados e biópsias, respectivamente (GEZUELE; DA ROSA, 2005). Para garantir o transporte do material a ser analisado, é necessário que a amostra seja coletada por *swabs* ou zaragatoas em meio Stuart e que os fragmentos de tecido sejam preservados em solução salina estéril. A semeação em meio de cultura deve ser realizada o mais rápido possível; caso não seja possível, a amostra deve ser armazenada a 4 ºC e por tempo inferior a 8-10 h (GREMIÃO et al., 2020b). Para o processamento, é necessário que o laboratório seja certificado como nível 2 de biossegurança, o que pode não estar disponível na localidade onde foi coletada a amostra. Além disso, a contaminação da amostra por outros fungos e bactérias bem como o crescimento lento característico, podem prejudicar ou causar atrasos nos resultados (RODRIGUES et al., 2022).

Por ser um fungo termodimórfico, o *Sporothrix* spp. pode assumir duas formas: micelial, quando incubado a 25 ºC e leveduriforme, quando incubado a 35-37 ºC (MORRIS-JONES, 2002). O micélio pode ser obtido em meios de cultura composto geralmente por ágar batata dextrose, ágar Sabouraud dextrose, podendo ser modificado com cicloheximida ou cloranfenicol sob incubação por cinco a sete dias (RODRIGUES; HAGEN; DE CAMARGO, 2022). Já a levedura deve ser semeada em meio enriquecido, como o ágar *brain heart infusion* (BHI), levando geralmente o mesmo tempo de incubação, porém é necessário observar a cultura por 30 dias, visto a possibilidade de contaminação (MORRIS-JONES, 2002).

Na morfologia macroscópica do micélio, as colônias filamentosas jovens têm coloração creme ou branca, com textura glaba e podem desenvolver pigmentos marrom a preto. Já as colônias maduras são tipicamente planas, coriáceas ou aveludadas e com pigmentação preta (FISHER, 2001). Microscopicamente são observadas hifas septadas, hialinas, ramificadas e finas (1 a 2 µm), como conidióforos surgindo em ângulo reto as hifas (Figura 2). Dois tipos de conídios podem ser produzidos, um menor do tipo hialino, de formato oval a piriforme e unicelular, com

paredes finas; e outro também unicelular, mas coloração marrom-escuro, com formato esférico ou oval e com paredes espessas (FISHER, 2001).

Na morfologia macroscópica da levedura, essas assumem coloração creme, marrom e textura lisa. Já avaliação micromorfológica, as células variam de fusiformes, globosas a ovais (2-6 µm), caracterizadas pela forma de charuto, com fixação delicada entre as células mães e células filhas (KWON-CHUNG; BENNETT, 1992).

2.5.2 Exame citopatológico

A avaliação por citopatologia é muito utilizada como acompanhamento terapêutico e evolutivo da doença e pré-diagnóstico, principalmente permitindo o início do tratamento antifúngico quando há dificuldades na realização da cultura micológica (GREMIÃO et al., 2020b). É um teste barato, não requer certificações de laboratório como ocorre no diagnóstico padrão, porém é necessário que o avaliador tenha ampla experiência na identificação do agente e que a lâmina com material a ser pesquisado tenha sido montada de modo eficaz (PEREIRA et al., 2011).

Para coloração da lâmina, os métodos por panótipo rápido (Romanowsky), tipo Gram e Giemsa são os mais utilizados para o diagnóstico da esporotricose (RASKIN; MEYER, 2011). Após este procedimento, a lâmina deve ser analisada usando lentes objetivas de pelo menos 40× ou preferencialmente de 100× de um microscópio óptico. Estruturas celulares semelhantes a leveduras são encontradas em grande quantidade, tendo formato ovalado, arredondado ou em charuto, com cerca de 3-5 µm de diâmetro e 5-9 µm de comprimento (PEREIRA et al., 2011). É importante ressaltar que mesmo que não seja observado estruturas condizentes ao *Sporothrix* spp. não é possível descartar a doença, principalmente em gatos sob tratamento em que pode ocorrer uma diminuição da carga fúngica no momento da coleta (MIRANDA et al., 2013).

2.5.3 Exame histopatológico

O exame histopatológico também pode ser utilizado como ferramenta diagnóstica preliminar ou de acompanhamento, porém neste caso o material biológico é coletado por meio de necropsia ou biópsia. A biópsia é realizada após sedação do

paciente, anestesia local com lidocaína 2% e antissepsia com álcool 70%, coletando a amostra das bordas ativas da lesão com auxílio de um *punch* (GREMIÃO et al., 2020b). O material de interesse é fixado em formol 10%, disposto em blocos de parafina, seccionados em micrótomo (5 µm) e corados com hematoxilina e eosina (HE) para observação de células inflamatórias e com metenamina de prata de Grocott-Gomori (GMS) ou ácido periódico de Schiff (PAS) para melhor visualização das leveduras do *Sporothrix* spp. (CARSON; HLADIK, 2009; DUNSTAN et al., 1986).

As lesões podem apresentar estruturas leveduriformes de formato oval, arredondado ou em charuto, acompanhadas por uma infiltração granulomatosa malformada com histiócitos pouco ativos e macrófagos predominantes (MIRANDA et al., 2013). Outras células de defesa como neutrófilos, linfócitos, eosinófilos e mastócitos podem ser visualizados. Com o início do manejo terapêutico, as lesões podem apresentar fibrose dérmica associada a infiltrado granulomatoso (SOUZA et al., 2018).

2.5.4 Teste sorológico

Os primeiros testes sorológicos para diagnóstico de esporotricose foram realizados em 1910, usando teste de aglutinação em tubo e fixação em complemento em pacientes humanos acometidos por *S. schenckii* (WIDAL; ABRAMI, 1912). Com passar dos anos, outros métodos foram testados, como o teste de aglutinação em lâmina, imunodifusão e anticorpo indireto fluorescente, revelando ferramentas como boa sensibilidade e que poderiam ser utilizadas de forma rápida e eficaz na rotina clínica (BLUMER et al., 1973). O primeiro teste sorológico baseado no ELISA foi descrito em 1980, no qual foi utilizado uma preparação da forma leveduriforme do *S. schenckii* para diagnóstico de esporotricose em humanos (SCOTT; MUCHMORE, 1989).

Com o propósito de avaliar interações antígeno-anticorpo detectáveis por meio de reações enzimáticas, é necessário escolher o tipo de ensaio a ser utilizado: direto, indireto, sanduíche e de captura (ENGVALL; PERLMANN, 1971). O ELISA indireto é o mais utilizado para o diagnóstico de esporotricose, tendo como princípio a detecção de anticorpos de pacientes expostos a doença (BERNARDES-ENGEMANN et al., 2005, 2015). Uma placa com 96 poços é sensibilizada com um antígeno já conhecido e é acrescentado os anticorpos a ser avaliados, estes chamados de primário. Ao

contrário do ELISA direto, é incluído um anticorpo secundário na reação, cujo é específico ao primeiro e promove mais especificidade ao teste. Por fim, é acrescentado um substrato cromogênico, promovendo uma reação colorimétrica avaliada por meio de espectrofotometria (CROWTHER, 2000).

Diversos tipos de antígenos foram estudados para o diagnóstico da esporotricose, seja na forma de preparações cruas e exoantígenos, proteínas recombinantes ou peptídeos sintéticos, porém ainda há poucos estudos (PARREIRAS DE JESUS et al., 2020). Em humanos, a literatura mais recente relata o desenvolvimento de um ELISA indireto utilizando um antígeno bruto a partir da forma micelial de *S. schenckii* para diagnóstico de esporotricose com 100% de sensibilidade e 98% de especificidade (ALVARADO et al., 2015). Em gatos, o uso do exoantígeno obtido a partir do *S. brasiliensis* promoveu um teste com 89% de sensibilidade e 82% de especificidade (BERNARDES-ENGEMANN et al., 2022). Todavia, o uso de antígenos obtidos de proteínas recombinantes ainda é pouco expressivo, com apenas um estudo em felinos e proveniente do *S. schenckii* cujo não é o agente etiológico primário da doença nesta espécie (FERNANDES et al., 2011).

3 Artigos

3.1 Artigo 1

Evaluation of recombinant *S. brasiliensis* protein in enzyme-linked immunosorbent assay in the serological diagnosis of feline sporotrichosis

Débora Matilde de Almeida; Laura de Vargas Maiocchi; Natasha Rodrigues de Oliveira; Camila Moura Lima; Martha Bravo Cruz Piñeiro; Odir Antonio Dellagostin; Sérgio Jorge; Márcia de Oliveira Nobre

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Evaluation of recombinant *S. brasiliensis* protein in enzyme-linked immunosorbent assay in the serological diagnosis of feline sporotrichosis

ALMEIDA, Débora Matilde de[1]; MAIOCCHI, Laura de Vargas [2]; DE OLIVEIRA, Natasha Rodrigues[2]; LIMA, Camila Moura[1]; PIÑEIRO, Martha Bravo Cruz[1]; SANZO, Gabriela Ladeira[1]; DELLAGOSTIN, Odir Antonio[2]; JORGE, Sérgio[1]; NOBRE, Márcia de Oliveira[1]

[1]Faculdade de Veterinária -Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul

[2] Centro de Desenvolvimento Tecnológico, Núcleo de Biotecnologia – Universidade Federal de Pelotas.

Resumo

Sporotrichosis is a zoonotic infection caused by fungi belonging to the *Sporothrix spp.* complex, with *S. brasiliensis* being the most prevalent etiological agent in affected animals, particularly cats. The gold standard for diagnosing sporotrichosis is the isolation and identification of *Sporothrix* species from clinical samples. Given the global increase in cases, there is a need for the development of an accurate, rapid, and cost-effective diagnostic test. The aim of this study was to develop a diagnostic test for feline sporotrichosis through an indirect ELISA using a recombinant *S. brasiliensis* chimera protein. The antigen was produced from immunogenic fragments of two *S. brasiliensis* proteins, SsEno and Gp70. Forty-eight feline serum samples were evaluated: 25 from cats diagnosed with sporotrichosis, 15 from healthy cats, and eight from other infectious diseases. The test demonstrated 88% sensitivity (95% CI: 70%-96%) and 100% specificity (95% CI: 85%-100%). The antigen application was satisfactory, resulting in a low-cost, rapid diagnostic test with promising potential for validation and further applications.

Keywords: ELISA; cats; recombinant protein; serodiagnosis.

1. Introduction

Sporotrichosis is a subcutaneous infectious disease caused by the dimorphic fungus of the *Sporothrix* spp. complex. Currently, four pathogenic species of clinical interest are considered, with *S. schenckii sensu stricto* reported worldwide and *S. luriei* being rarer (DE BEER; DUONG; WINGFIELD, 2016). The *S. globosa* specie is reported in cases of human sporotrichosis on the Asian continent, primarily in Japan, India, Malaysia, and China (MOUSSA et al., 2017). Meanwhile, *S. brasiliensis* is the most virulent species, causing outbreaks primarily in Brazil, where it has been reported in 25 states, with Rio de Janeiro being the epicenter of the disease (DE OLIVEIRA BENTO et al., 2021; FALCÃO et al., 2020).

Felines are the most affected species, primarily by *S. brasiliensis*. The animals become infected with the fungus present in their natural habitat, through their habit of scratching tree bark and contact with organic matter, or through scratches and bites during intraspecies aggression (RAMÍREZ-SOTO et al., 2018). The lesions are usually disseminated, with a nodular-exudative nature, and are found on the face, ears, nose, limbs, and posterior regions (GREMIÃO et al., 2015).

The definitive diagnosis is established through the isolation of *Sporothrix spp.* via mycological culture and the observation of yeast cells in the morphological characterization (DE MIRANDA et al., 2018). Other methods can also be used as follow-up or pre-diagnostic strategies, such as cytological examination by imprint or swab and histopathological examination by biopsy or necropsy (GREMIÃO et al., 2020b; MIRANDA et al., 2013; PEREIRA et al., 2011). However, due to the slow growth of the fungus in its yeast form and the requirement that the laboratory be certified as biosafety level 2 for a definitive diagnosis, there may be delays in obtaining results, which can negatively impact patient management and treatment (GREMIÃO et al., 2020b; SILVA et al., 2018).

Given the challenges associated with conventional diagnosis, other alternatives are being applied, such as the use of PCR or serological tests, including immunodiffusion, tube agglutination, and ELISA (BLUMER et al., 1973; FERNANDES et al., 2011). The use of indirect enzyme-linked immunosorbent assay (ELISA) as a diagnostic tool is based on the potential for a faster test compared to the standard method, with high sensitivity and specificity (ALVARADO et al., 2015; PORTUONDO et al., 2022). There is only one study using *S. brasiliensis* in its mycelial form as an

antigen in ELISA diagnosis for felines. (RODRIGUES et al., 2015b). The others use exoantigens or recombinant proteins obtained from *S. schenckii* (BERNARDES-ENGEMANN et al., 2022; FERNANDES et al., 2011; RODRIGUES et al., 2015b). In humans, indirect ELISA has been used both for diagnosis and therapeutic monitoring of patients, regardless of the clinical form or recurrence of episodes (ALMEIDA-PAES et al., 2007; BERNARDES-ENGEMANN et al., 2005). Considering the challenges in diagnosing feline sporotrichosis, the zoonotic risk, and the increasing number of cases worldwide, there is a need to research new diagnostic assays that are both effective and rapid. Thus, the aim of this study was to develop a diagnostic test for sporotrichosis in felines using indirect ELISA with a recombinant *S. brasiliensis* protein as the antigen.

2. Materials and Methods

2.1 Feline Serum

Twenty-five feline serum samples were selected from cats diagnosed with sporotrichosis based on the presentation of clinical signs compatible with the disease, agent isolation, and morphological characterization. The animals were previously classified according to sex (8 females and 17 males), whether they were affected by feline leukemia virus (FeLV) (n = 3; 12%) and/or feline immunodeficiency virus (FIV) (n = 7; 28%). Regarding the general condition and presentation of lesions, the cats were classified into groups according to the criteria established by Schubach et al (2004) and Miranda et al (2013), as follows: L1 – lesions in only one location (n = 7; 28%); L2 – lesions in two non-contiguous locations (n = 1; 4%); L3 – lesions in three or more non-contiguous locations (n = 17; 68%). Concerning the general condition, the cats were classified as S1 – good general condition, without systemic signs (n = 15; 60%); S2 – fair general condition, with mild systemic signs (n = 6; 24%); S3 – delicate general condition, with moderate systemic signs (n = 4; 16%); S4 – critical general condition, with severe systemic signs (n = 0; 0%). The systemic signs of interest included dehydration, apathy, anorexia, pale mucous membranes, hyperthermia, or hypothermia, among others.

To determine the specificity of the test, eight serum samples from felines with other infectious diseases, specifically FeLV (n = 7) and FIV (n = 1), previously diagnosed by immunochromatography, were used. The samples were kindly provided by an animal serum bank located in Pelotas, RS. Additionally, 15 serum samples from

healthy felines, without clinical signs of any disease and negative for retroviruses, were selected. All animal serum samples used in this study were obtained from a serum bank of previously completed projects approved by the Animal Ethics and Experimentation Committee (CEEA) of the Veterinary College at UFPel (30742/2018) (24343019.9.0000.5317).

2.2 Selection of Amino Acid Sequences

The protein named rChiGp70_Eno was constructed from the coding sequences of the Gp70 protein and epitopes of *S. brasiliensis* 5110 enolase. The coding sequences used are deposited in GenBank (63678217 and 63673753, respectively). The selected epitopes from rSsEno were identified by Portuondo et al (2022), who observed the potential for their use as a vaccine antigen due to their characteristic of not triggering an autoimmune response in felines. For the construction of the chimera, a region of Gp70 with the highest concentration of B-cell epitopes was selected, and this region was fused with six rSsEno epitopes via a flexible linker. The amino acid sequence of the chimera was subjected to structural modeling using the trRosetta and I-TASSER software. The best model for the generated structures was refined using the ModRefiner algorithm, and quality analysis was performed with the Qmean6 and ModFOLD4 servers. The stoichiometry of the refined model was assessed using Ramachandran plot analysis (RAMACHANDRAN; RAMAKRISHNAN; SASISEKHARAN, 1963) through the online Ramachandran plot Server (https://zlab.umassmed.edu/bu/rama/), resulting in a final protein of approximately 51.59 kDa. After thorough analysis with bioinformatics software and structural modeling, the gene was chemically synthesized by a specialized company (GenOne) and provided in the pET28a vector.

2.3 Cloning, Expression, and Purification of the Chimeric Protein

The plasmid of interest was introduced via calcium chloride heat shock transformation using E. coli BL21(DE3) Star as the expression strain. The transformed product was inoculated into 500 ml of LB medium with kanamycin (50 µg/ml) and cultured overnight at 37°C with shaking at 180 rpm. A 0.5 ml aliquot of the culture was re-incubated under the same shaking and temperature conditions until the bacterial growth reached the exponential phase, indicated by an $D.O._{600\ nm}$ between 0.6 and 0.8. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM/ml,

and the culture was incubated for 3 hours at 37°C with shaking at 180 rpm. Pre- and post-induction samples were centrifuged at 10,000 × g for two minutes, and the resulting pellets were frozen for later expression analysis. The remaining culture was centrifuged (7,000 g × 10 min), and the pellet was resuspended in 1× PBS and sonicated as previously described (MAIA et al., 2022). Purification was carried out using affinity chromatography with a Ni-Sepharose HiTrap 1 ml column. Fractions containing the purest proteins were dialyzed against 1× PBS buffer containing 0.05% Triton X-100 and decreasing concentrations of urea. Protein expression was confirmed through one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4 Western blot

The antigenic potential of the recombinant protein was assessed by Western blot using a pool of positive sera for sporotrichosis, as previously described (MAIA et al., 2022). Briefly, the recombinant protein was subjected to 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with a pool of feline sera positive for sporotrichosis (1:500) and monoclonal anti-6x histidine antibody conjugated with peroxidase (1:5000; Sigma Aldrich). Anti-cat IgG conjugated with peroxidase was used as the secondary antibody.

2.5 ELISA indireto

Polystyrene microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, NY, USA) with 96 wells were coated in triplicate with 200 ng of the rChiGp70_Eno protein per well diluted in 50 mM Carbonate-Bicarbonate buffer (pH 9.8) and incubated at 4°C overnight. After this period, the plates were washed three times with 200 µl per well of PBS-T and blocked with 200 µl of blocking solution (non-fat dry milk) at 5% in PBS-T for 2 hours at 37°C. After washing four times with PBS-T, the plates were incubated for 1 hour at 37°C with sera from cats diagnosed with sporotrichosis at a 1:50 dilution in 2.5% non-fat dry milk in PBS-T. The plates were washed three times with PBS-T and then incubated with horseradish peroxidase-conjugated anti-feline IgG (Sigma-Aldrich) diluted 1:5000 in PBS-T for 1 hour at 37°C. Finally, the plates were washed five times with PBS-T, and the reaction was developed by adding a solution containing 0.004 g of o-phenylenediamine dihydrochloride in 10 ml of citrate phosphate buffer (pH 4.0) and 15 µl of 30% H_2O_2 for 15 minutes at room

temperature in the dark. The reaction was stopped by adding 50 µl of 3% H_2SO_4 per well, and absorbance was measured using an ELISA plate reader (Agilent BioTek 800 TS Absorbance Reader, Santa Clara, CA, USA) at 492 nm. All samples were evaluated in triplicate.

2.6 Statistical Analysis

The obtained data were tabulated and processed using GraphPad Prism 5.0 (La Jolla, CA, USA). The analysis will include evaluation of the cutoff point, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), overall efficiency, and the receiver operating characteristic (ROC) curve.

The cutoff point was defined as the arithmetic mean plus 2 standard deviations of the sera from healthy cats. Sensitivity, which measures how well the test can correctly identify sera from felines with sporotrichosis, was defined by the ratio of true positives (TP) to the sum of true positives and false negatives (TP + FN). Specificity, which measures the test's ability to correctly identify sera from healthy animals, was defined by the ratio of true negatives (TN) to the sum of true negatives and false positives (TN + FP). The positive predictive value (PPV), which refers to the probability that a positive result indicates an animal with sporotrichosis, was defined by the ratio of true positives to the sum of true positives and false positives (TP / (TP + FP)). The negative predictive value (NPV), which refers to the probability that a negative result indicates a healthy animal, was defined by the ratio of true negatives to the sum of true negatives and false negatives (TN / (TN + FN)). The overall efficiency of the test was defined by the ratio of the sum of true positives and true negatives to the sum of true positives, true negatives, false positives, and false negatives ((TP + TN) / (TP + TN + FP + FN)). The confidence interval and ROC curve were included to indicate the reliability and performance of the test, respectively. To correlate absorbances among different lesion classifications and with general condition assessments, the non-parametric Mann-Whitney test was used.

3. Results

3.1 Characterization and Antigenicity of the Recombinant Protein

The Western blot results using the anti-6×Histidine antibody revealed a protein band of the expected size (~51.9 kDa). The chimeric protein was also specifically

recognized by the pool of sera from infected felines (Figure 1), confirming the retention of immunogenic epitopes in the construct.

3.2 Performance of the ELISA

Of the 48 samples analyzed, 22 were correctly classified as true positives, with only three being false negatives. All 15 samples from healthy cats were accurately identified as true negatives, as were the eight samples from cats diagnosed with other infectious diseases (Table 1). The cutoff point established as the mean plus two standard deviations of the sera from negative cats was 0.022 (Figure 2). Consequently, the performance of the assay was 88% (95% CI: 70%-96%) sensitivity, 100% (95% CI: 85%-100%) specificity, 88.5% negative predictive value, 100% positive predictive value, and 93.8% overall efficiency. The area under the ROC curve was 0.964 ± 0.025 (95% CI: 0.9148 – 1.000), as shown in Figure 3.

4. Discussion

As an emerging disease with significant zoonotic potential, sporotrichosis has been the focus of studies due to large outbreaks in Brazil, particularly in Rio de Janeiro (ALVAREZ; OLIVEIRA; PIRES, 2022; RABELLO et al., 2022). Due to sanitary conditions and the increasing feline population, the expansion of the endemic disease has been accelerated. However, the implementation of sanitary surveillance measures for disease control has not kept pace (SCHUBACH; SCHUBACH; BARROS, 2005). In the epidemiological context, cats play a crucial role in the transmission of the disease, as many are not housed indoors and have the habit of sharpening their claws on trees, making them susceptible to carrying the fungus. They can transmit the fungus through scratches or bites during intraspecies fights or accidents involving humans (DE OLIVEIRA BENTO et al., 2021). As the primary agent of feline sporotrichosis, *S. brasiliensis* is a highly virulent species and is responsible for the majority of cases in the country (GREMIÃO et al., 2020a; RODRIGUES et al., 2014). To combat the disease, diagnostic tests are being developed based on serological evaluation, using increasingly specific antigens in the form of synthetic peptides, exoantigens, and recombinant proteins (BERNARDES-ENGEMANN et al., 2022; FERNANDES et al., 2011).

In this study, we describe the development and evaluation of an indirect ELISA using a recombinant protein from *S. brasiliensis* for the serological diagnosis of feline sporotrichosis. Analysis of 48 feline serum samples yielded 88% sensitivity and 100% specificity with the developed test. In a similar study, the use of an exoantigen obtained from an *S. brasiliensis* CFP 817 strain achieved 87% sensitivity and 100% specificity, supporting our results (BERNARDES-ENGEMANN et al., 2022). In the author's opinion, having worked with both forms, the recombinant protein allows for better standardization of the ELISA compared to crude antigen, especially if the laboratory where the test was developed has more advanced biotechnological resources.

On the other hand, when using a purified protein from *S. schenckii* for the serological detection of cats with sporotrichosis, the obtained results were 90% sensitivity, which is higher than the result found in our study. However, the specificity was lower, at 96% (FERNANDES et al., 2011). When combining four strains of *S. schenckii* and *S. brasiliensis* processed as exoantigens, the serological test using ELISA achieved 100% sensitivity and specificity (RODRIGUES et al., 2015b). *Despite the limited number of studies, these results reveal that it is possible to use different antigens for the diagnosis of feline sporotrichosis, as there is antigenic epitope sharing between S. schenckii and S. brasiliensis* (MARIMON et al., 2006, 2007; RODRIGUES et al., 2015a).

In our study, three samples were evaluated as false negatives. Reviewing the history of the cats from which these samples were collected, all were classified as having a good general condition and no clinical signs (S1); however, two had lesions in three or more locations on the body (L3), and only one had a lesion in a single location (L1). Correlating the absorbances of the different lesion classifications (L1 vs. L2; L1 vs. L3; L2 vs. L3) showed no significant differences in any of the groups ($p = 0.24; 0.07; 0.76$). Similarly, there were no significant differences when correlating absorbances with different general conditions. With limited data on the application of ELISA in the clinical monitoring of cats with sporotrichosis, further studies on clinical staging and disease progression are needed (BAPTISTA et al., 2020).

Our study has some limitations, primarily due to the number of samples analyzed, both from diseased and healthy cats. Although the sample size is consistent with other findings in the literature, the small number of samples may significantly impact the sensitivity of the test. Additionally, due to the low number of affected animals and the consequent inability to collect samples, it was not possible to include

heterologous sera in the ELISA development. Thus, the assessment of potential cross-reactions with other fungal diseases such as cryptococcosis and histoplasmosis may have been compromised. Despite these limitations, our evidence suggests that the use of the recombinant *S. brasiliensis* protein as an antigen was effective in developing the ELISA for diagnosing feline sporotrichosis, demonstrating high sensitivity and specificity in serological analyses.

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Competing Interests

The authors declare there are no competing interests.

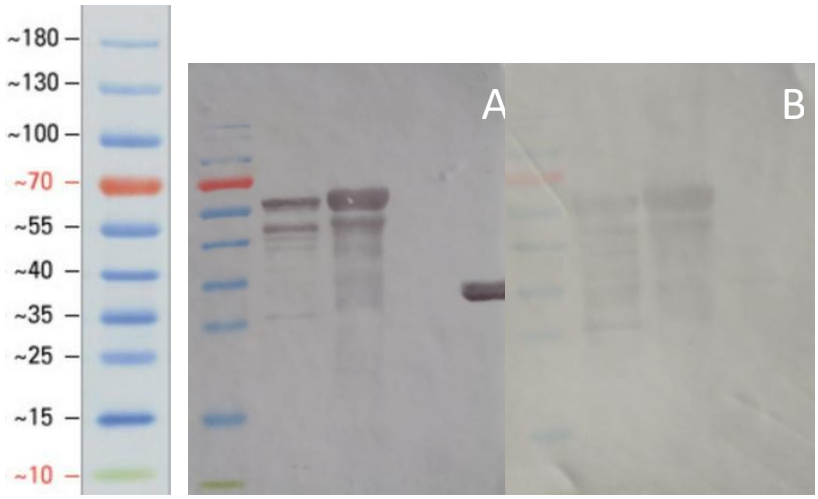

Figure 1 – Antigenic characterization of the recombinant chimera. A, Western blot with anti-6×His antibody conjugated to peroxidase; B, Western blot with pool of convalescent feline sera. M, molecular weight marker; 1, protein expressed post-induction; 2, purified protein; 3, negative control BSA; 5, positive control rLipL32.

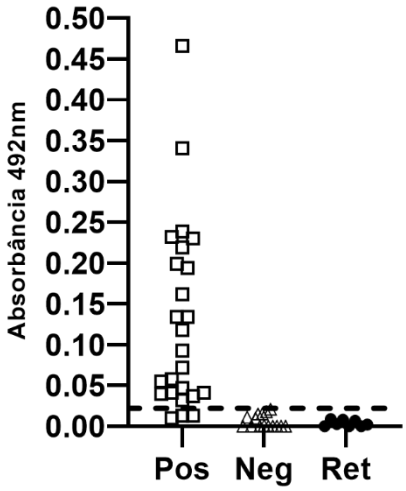

Figure 2 – Absorbances obtained from the evaluation of sera from cats diagnosed with sporotrichosis (Pos), healthy cats (Neg), and cats with other infectious diseases (Ret) using the rChiGp70_Eno protein as the antigen in the ELISA. The dashed line represents the cutoff point of 0.022.

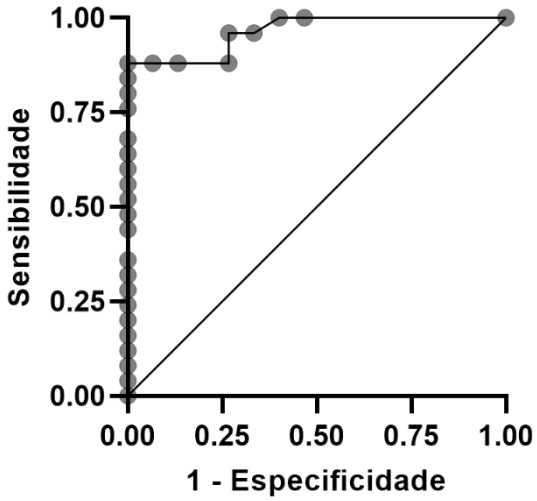

Figure 3 – ROC curve for the ELISA using the rChiGp70_Eno protein as the antigen. The area under the curve was 0.964, representing the accuracy of the serological test.

Table 1 – 2x2 Table for the results obtained from the ELISA. The reference test tab includes the negative samples and those with other infectious diseases.

		Reference Standard		
		Positive	Negative	Total
ELISA	Positive	22	0	22
	Negative	3	23	26
	Total	25	23	48

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3.2 Artigo 2

Diagnostic Accuracy of ELISA for Sporotrichosis: A Systematic Review and Meta-Analysis

Débora Matilde de Almeida, Eduarda Santos Bierhals, Gabriel Peres Messenburger, Sérgio Jorge, Márcia de Oliveira Nobre

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Precisión diagnóstica del ELISA para la esporotricosis: revisión sistemática y metaanálisis

Título corto: Precisión diagnóstica del ELISA para la esporotricosis

Diagnostic Accuracy of ELISA for Sporotrichosis: A Systematic Review and Meta-Analysis

Short title: Diagnostic Accuracy of ELISA for Sporotrichosis

Débora Matilde de Almeida[a], Gabriel Peres Messenburger[b], Eduarda Santos Bierhals[a], Sérgio Jorge[a*], Márcia de Oliveira Nobre[a]

[a)] College of Veterinary, Universidade Federal de Pelotas (UFPel), Brazil

[b)] College of Nutrition, Universidade Federal de Pelotas, Brazil

*Corresponding author: Universidade Federal de Pelotas, Campus Capão do Leão, Faculdade de Veterinária prédio 1, Laboratório ClinPet Sala 2, Capão do Leão-RS, Brazil, Postal Code: 96160-000, Tel.: +55 53 3284 3830.

E-mail address: sergiojorgevet@hotmail.com

Abstract

Background: Sporotrichosis is a subcutaneous mycosis caused by traumatic inoculation of dimorphic fungi from the genus *Sporothrix*. Traditional diagnostic methods, including direct microscopy and fungal culture, are time-consuming, require specialized expertise, and may yield false-negative results.

Aim: This systematic review and meta-analysis aimed to evaluate the diagnostic accuracy of the enzyme-linked immunosorbent assay (ELISA) in detecting sporotrichosis in humans and animals.

Methods: A comprehensive literature search was conducted across PubMed, LILACS, EMBASE, Scopus, Web of Science, ScienceDirect, CAPES Periodicals, and the Cochrane Library using both free-text terms and MeSH descriptors. The methodological quality of included studies was assessed using the QUADAS-2 tool. Pooled sensitivity and specificity were estimated using a random-effects model. Secondary outcomes included the positive and negative likelihood ratios and the diagnostic odds ratio (DOR).

Results: Twelve studies met the inclusion criteria, comprising 1,612 samples (1,350 human and 262 feline). ELISA demonstrated a pooled sensitivity of 91% (95% CI: 85–94) and specificity of 90% (95% CI: 86–93), with a DOR of 94.2 (95% CI: 46.9–189.1). Subgroup analysis revealed higher diagnostic performance in feline samples, particularly when crude antigen extracts and combined fungal forms were used.

Conclusions: ELISA exhibits high diagnostic accuracy for sporotrichosis in both human and veterinary contexts. Its performance, especially in feline hosts, supports its potential role as a reliable diagnostic alternative to conventional methods.

Keywords: Indirect diagnosis, mycosis, *Sporothrix brasiliensis, Sporothrix schenckii*, zoonosis.

Resumen

Antecedentes: La esporotricosis es una micosis subcutánea causada por la inoculación traumática de hongos dimórficos del género *Sporothrix*. Los métodos diagnósticos tradicionales, como la microscopía directa y el cultivo fúngico, requieren mucho tiempo, demandan conocimientos especializados y pueden arrojar resultados falsos negativos.

Objetivos: Esta revisión sistemática y metaanálisis tuvo como objetivo evaluar la precisión diagnóstica del ensayo por inmunoabsorción ligado a enzimas (ELISA) para la detección de esporotricosis en humanos y animales.

Métodos: Se realizó una búsqueda bibliográfica exhaustiva en PubMed, LILACS, EMBASE, Scopus, Web of Science, ScienceDirect, Periódicos CAPES y Cochrane Library utilizando términos libres y descriptores MeSH. La calidad metodológica de los estudios incluidos se evaluó mediante la herramienta QUADAS-2. La sensibilidad y especificidad agrupadas se calcularon utilizando un modelo de efectos aleatorios. Los desenlaces secundarios incluyeron las razones de verosimilitud positiva y negativa, así como la razón de odds diagnóstica (DOR).

Resultados: Doce estudios cumplieron los criterios de inclusión, abarcando un total de 1.612 muestras (1.350 humanas y 262 felinas). El ELISA mostró una sensibilidad agrupada del 91% (IC 95%: 85–94) y una especificidad del 90% (IC 95%: 86–93), con una DOR de 94,2 (IC 95%: 46,9–189,1). El análisis por subgrupos evidenció un mayor rendimiento diagnóstico en muestras felinas, especialmente cuando se emplearon extractos antigénicos crudos y formas fúngicas combinadas.

Conclusión: El ELISA presenta una alta precisión diagnóstica para la esporotricosis en contextos tanto humanos como veterinarios. Su desempeño, especialmente en huéspedes felinos, respalda su aplicación como una alternativa diagnóstica confiable frente a los métodos convencionales.

Palabras clave: diagnóstico indirecto; micosis; *Sporothrix brasiliensis; Sporothrix schenckii,* zoonosis

Introduction

Sporotrichosis is a chronic subcutaneous mycosis caused by thermally dimorphic fungi of the genus *Sporothrix*. As a cosmopolitan disease, sporotrichosis holds particular importance due to its global distribution and status as a neglected mycosis, posing a significant public health challenge (1). While prevalent worldwide, it has gained particular prominence in Latin America, where it is the most common implantation mycosis. High-endemic areas include Brazil, Colombia, Peru, and Venezuela, underscoring the urgent need for attention to its growing impact on both regional and international health landscapes (2–4).

Infection typically results from traumatic inoculation of fungal elements via contaminated plant material or soil, or through animal bites and scratches (5–8). Notably, domestic cats play a central role in the transmission dynamics of zoonotic sporotrichosis due to their susceptibility and close contact with humans. Risk factors such as outdoor roaming, inter-cat aggression (especially in unneutered males), and clawing behavior enhance their potential as reservoirs (9–11).

This genus exhibits wide genomic diversity, and at least four of its species have been identified as agents of human or animal infection. Among these, *Sporothrix schenckii sensu stricto, S. brasiliensis, S. globosa*, and *S. luriei* are considered the most clinically relevant species, while *S. mexicana, S. pallida*, and *S. chilensis* are more frequently found in the environment, with rare cases of causing disease (12–15).

Current diagnostic standards rely on direct microscopic examination and fungal culture from clinical specimens (16–18). While cultures allow for species-level identification, the process is time-consuming, technically demanding, and frequently hampered by low fungal burden in human lesions, which can delay diagnosis and treatment (19,20). Serological assays have gained attention as promising alternatives, offering higher sensitivity and faster turnaround (21). Various antigenic targets—including fungal cell wall components, recombinant proteins, and synthetic peptides—have been investigated for their diagnostic utility (21,22). Among

these, the enzyme-linked immunosorbent assay (ELISA) has emerged as the most extensively evaluated, demonstrating high diagnostic potential in both human and veterinary settings (23,24).

The objective of this systematic review and meta-analysis is to critically evaluate and synthesize the available evidence regarding the performance of ELISA for the diagnosis of sporotrichosis. By quantifying diagnostic accuracy across studies and identifying sources of heterogeneity, we aim to support the development and implementation of more effective serological tools for the diagnosis and epidemiological surveillance of this neglected fungal disease.

Methods

Study Design

This systematic review and meta-analysis was conducted in accordance with the Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy and reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (25,26). The protocol was prospectively registered in the PROSPERO database (*CRD42023410139*).

Research Question

The research question was framed using the PIRD (Population, Index test, Reference standard, Diagnosis) structure: “Is the enzyme-linked immunosorbent assay (ELISA) an effective diagnostic tool for detecting sporotrichosis in humans and animals?”

Data sources and searches

A comprehensive literature search was performed across eight databases: PubMed, LILACS, EMBASE, Scopus, Web of Science, ScienceDirect, CAPES Periodicals, and the Cochrane Library, covering all publications up to January 2024. The search combined free-text terms

with controlled vocabulary (MeSH) related to sporotrichosis and ELISA. The complete search strategy is provided in Supplementary Table S1.

Eligibility criteria

We included diagnostic accuracy studies: cross-sectional, case-control, cohort, and clinical trials reporting ELISA-based serodiagnosis of sporotrichosis in humans or animals. Studies were required to present sufficient data to construct 2×2 contingency tables (i.e., true positives [TP], false positives [FP], false negatives [FN], and true negatives [TN]), either directly or derivable from the reported results. Studies that were duplicates, studies that evaluated sporotrichosis but not in relation to an ELISA diagnostic test, as well as narrative and systematic reviews or meta-analyses, were excluded from the analysis.

Study selection

Following the completion of searches in multiple databases, a manual review was performed to eliminate duplicate articles. Two independent reviewers (DMA and ESB) thoroughly evaluated the titles, abstracts, and full texts of the articles. Any disagreements that arose during the review process were resolved through consensus. In the event that a consensus could not be reached, a third reviewer was consulted to resolve the disagreement. Additionally, a manual search of the bibliographic references of all studies included in this systematic review was conducted to identify any additional articles that were not uncovered through electronic searching. The entire study selection process was facilitated using the Rayyan platform (27).

Assessment of methodological quality

The quality of the studies included in this analysis was assessed using the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) (28). The QUADAS-2 tool facilitates the assessment of the quality of studies by evaluating the risk of bias and the applicability of results across four domains: participant selection, index test, reference standard,

and flow and timing. Two independent reviewers (DMA and GPM) conducted the quality assessment of the studies, and any disagreements were resolved through consensus. In the event that consensus could not be reached, a third reviewer was consulted to resolve the issue.

Data analysis

In this meta-analysis, we extracted the true positive (TP), false positive (FP), false negative (FN), and true negative (TN) values from each included study. These data were used to calculate sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR).

RevMan 5.4.1 and R (29) were employed for statistical analyses in this study. RevMan was used to generate forest plots, assess the methodological quality of the included studies using QUADAS-2, and to construct a summary receiver operating characteristic (SROC) plot. On the other hand, R was used to calculate the pooled diagnostic accuracy measures, including sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, and diagnostic odds ratio.

These parameters were pooled using the random-effects model (30). This model accounts for the potential heterogeneity among studies, as it assumes that the studies were drawn from populations that exhibit differences that may impact diagnostic accuracy. The assumption was made that the diagnostic accuracy of the test varied between studies, and the degrees of accuracy were randomly distributed around a central value.

Investigations of heterogeneity

The magnitude of heterogeneity was interpreted using a classification of I^2 values, with values of 25%, 50%, and 75% being considered to indicate low, medium, and high levels of heterogeneity, respectively (31). Sensitivity analyses were conducted to explore potential sources of heterogeneity. In addition to the overall analysis that included all studies, subgroup

analyses were performed based on different population characteristics and variations in diagnostic test characteristics.

Results

Search Results

The initial database search yielded 336 records. After the removal of duplicates (n = 188), 148 titles and abstracts were screened. Of these, 128 were excluded based on the eligibility criteria. Full-text evaluation of the remaining 20 studies resulted in the exclusion of 8 articles: 2 reviews, 2 conference abstracts, and 4 studies that did not evaluate ELISA for sporotrichosis. A total of 12 studies were ultimately included in the qualitative and quantitative synthesis (Figure 1).

Studies characteristics

The 12 included studies encompassed a total of 1,612 serum samples: 1,350 human and 262 felines. These studies were conducted between 2005 and 2023 in Brazil, Argentina, and Venezuela. Eight studies evaluated human serum, and four evaluated feline serum. Of the total samples, 682 were confirmed positive for *Sporothrix* spp., 576 were classified as heterologous controls (e.g., infected with other diseases), and 354 samples were from healthy individuals. The characteristics of the included studies are summarized in Table 1.

Methodological Quality Assessment

In order to generate the QUADAS-2 graphs and summaries, RevMan 5.4.1 was employed, in which the methodological quality assessment was presented in Figure 2. Here, A is the risk of bias and applicability concerns summary, and B refers to the risk of bias and applicability concerns graph. Most studies showed unclear risk of bias in the domains of patient selection and index test. Two studies presented high risk of bias in the reference standard domain, and two showed unclear bias in the flow and timing domain.

According to the risk of bias analysis, all studies were classified as having an unclear risk of bias in the domains of patient selection and index test (21,23,24,32–38). In the reference standard domain, two studies were classified as having a high risk of bias, one study was classified as having an unclear risk (33,34), while the remaining studies had a low risk of bias. In the flow and timing domain, two studies were classified as having an unclear risk of bias, whereas the rest were classified as having a low risk of bias. In the applicability concerns section, all studies were classified as having low concern in the patient selection and index test domains (34,35). However, in the reference standard domain, two studies were classified as having unclear concern, while the rest were classified as having low concern (34,35).

Pooled Diagnostic Accuracy

Across all 12 studies, the pooled sensitivity and specificity of ELISA were 91% (95% CI: 85–94; I^2 = 67.7%) and 90% (95% CI: 86–93; I^2 = 67.7%), respectively. Forest plots illustrating individual and pooled sensitivity/specificity values, as well as the 2×2 diagnostic contingency data, are shown in Figure 3. The pooled positive likelihood ratio (PLR) was 8.84 (95% CI: 6.17–12.68), and the negative likelihood ratio (NLR) was 0.08 (95% CI: 0.05–0.13) (Figure S2). The pooled diagnostic odds ratio (DOR) was 94.2 (95% CI: 46.9–189.1), indicating high overall test performance (Figure 4). The SROC curve is presented in Figure 5.

Subgroup Analyses

Subgroup analysis by host species revealed that ELISA performed better in feline samples. In cats, the pooled sensitivity was 92% (95% CI: 84–97; I^2 = 26.0%), and specificity was 96% (95% CI: 92–98; I^2 = 0%). In contrast, for human samples, sensitivity was 90% (95% CI: 83–94; I^2 = 73.8%), and specificity was 88% (95% CI: 83–91; I^2 = 70.7%) (Figure S1). The DOR was markedly higher in feline studies (406.8; 95% CI: 122.5–1,351.8) compared to human studies (57.8; 95% CI: 29.2–114.6), supporting higher diagnostic discrimination in veterinary

applications. Subgroup analyses based on ELISA characteristics (Table 2) indicated that crude antigen extracts yielded higher sensitivity and specificity than recombinant proteins. Furthermore, assays incorporating both fungal forms (mycelial and yeast) showed better diagnostic performance than those using a single form. Tests targeting *S. schenckii* displayed lower diagnostic accuracy, although this may reflect older taxonomic classifications preceding the phylogenetic redefinition of the *Sporothrix* complex.

Discussion

This systematic review and meta-analysis is the first to specifically evaluate the diagnostic performance of ELISA for sporotrichosis in both humans and animals. Across 12 studies encompassing 1,612 samples, ELISA demonstrated high overall accuracy, with pooled sensitivity and specificity of 91% and 90%, respectively. These findings support the utility of ELISA as a reliable, non-invasive diagnostic alternative to traditional mycological methods.

The inclusion of both human and feline populations reflects the zoonotic nature of sporotrichosis, particularly in regions where domestic cats play a central epidemiological role (2,5,8). Cats are not only highly susceptible but also serve as major sources of transmission to humans. The superior diagnostic performance observed in feline samples (sensitivity: 92%; specificity: 96%) highlights the test's value in veterinary settings, where rapid, accurate diagnosis can aid in outbreak control and public health surveillance.

The risk of bias across studies was generally low; however, uncertainties remain in some domains. In particular, the lack of clarity in patient selection procedures and blinding of the index test raises concern. Knowledge of the reference standard results during ELISA testing may introduce observer bias, potentially inflating diagnostic accuracy. Future studies should address these methodological gaps by ensuring appropriate blinding and standardizing sample selection procedures. Two studies demonstrated high risk of bias in the reference standard domain, raising questions about the consistency and validity of culture-based confirmation

(34,35). Given the potential for false-negative cultures in human samples, overestimation or underestimation of ELISA performance may occur depending on how reference standards were applied.

Heterogeneity was considered moderate to high in the overall analysis; however, this decreased when the feline population was analysed separately. Notably, both specificity and sensitivity exhibited low heterogeneity. When study results are more consistent, there is increased confidence in the application of ELISA in feline patients. When we analysed the studies according to the population they examined, some interesting results emerged. The diagnostic odds ratio of the feline group was considerably higher than that of the human group. Additionally, the overall sensitivity and specificity of the feline group were higher.

In the subgroup analysis, our findings demonstrated that the crude extract exhibited superior sensitivity and specificity. However, a detailed review of the literature revealed a lack of consensus regarding the terminology used for exoantigens and crude extracts. This discrepancy is evident as different authors employed the same methodology for antigen extraction (39,40), but utilised varying terminologies. Consequently, when the two terms are aggregated, the results surpass those of other antigen types.

When analysing the fungal form, the literature presents a casual balance in the number of tests, as well as high sensitivity and specificity. However, for the most part, there is inconsistency between studies and their results, which reduces confidence. Therefore, it is speculated that more studies should be conducted, particularly evaluating different correlations of fungal forms within a single species or among multiple species. Our results indicated that the combination of two fungal forms was superior to individual use in both sensitivity and specificity. Among the individual species, *Sporothrix schenckii* exhibited the poorest overall performance. It is important to note that this result should be interpreted with caution, as most studies involving

S. schenckii were conducted prior to the phylogenetic analysis of the calmodulin-encoding protein, which resulted in the identification of new *Sporothrix* spp (41,42).

In terms of the diagnostic odds ratio, it provides significant advantages in the meta-analysis of diagnostic studies by combining results from various studies into summary estimates with enhanced precision (43). It is a statistical measure that evaluates the discriminative power of a diagnostic test by calculating the ratio of the odds of a positive test result among individuals with the target condition to the odds of a positive test result among those without the condition (44). Our study found a DOR of 94.20 (Figure 4), indicating excellent performance of this measure.

We found that ELISA tests demonstrate excellent overall accuracy for diagnosing sporotrichosis. Our findings are consistent with those of a meta-analysis published in 2020 that analysed various types of serological tests for diagnosing sporotrichosis and arrived at similar conclusions (45). Our study has some limitations, primarily due to the low number of serological samples analysed, particularly since the majority of samples were from humans, with only a small proportion from cats. To enhance the quality of studies and, consequently, the certainty of the evidence, further research is needed, especially studies that evaluate ELISA using blinded testing, without knowledge of the reference standard results, and with a larger sample size. Nevertheless, the evidence indicates that ELISA can be a valuable tool to assist in the diagnosis of sporotrichosis. We found that ELISA exhibits excellent overall accuracy in diagnosing sporotrichosis in both humans and animals, reinforcing their potential as a reliable diagnostic tool in clinical practice.

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Conflicts of interest

All authors have nothing to disclose. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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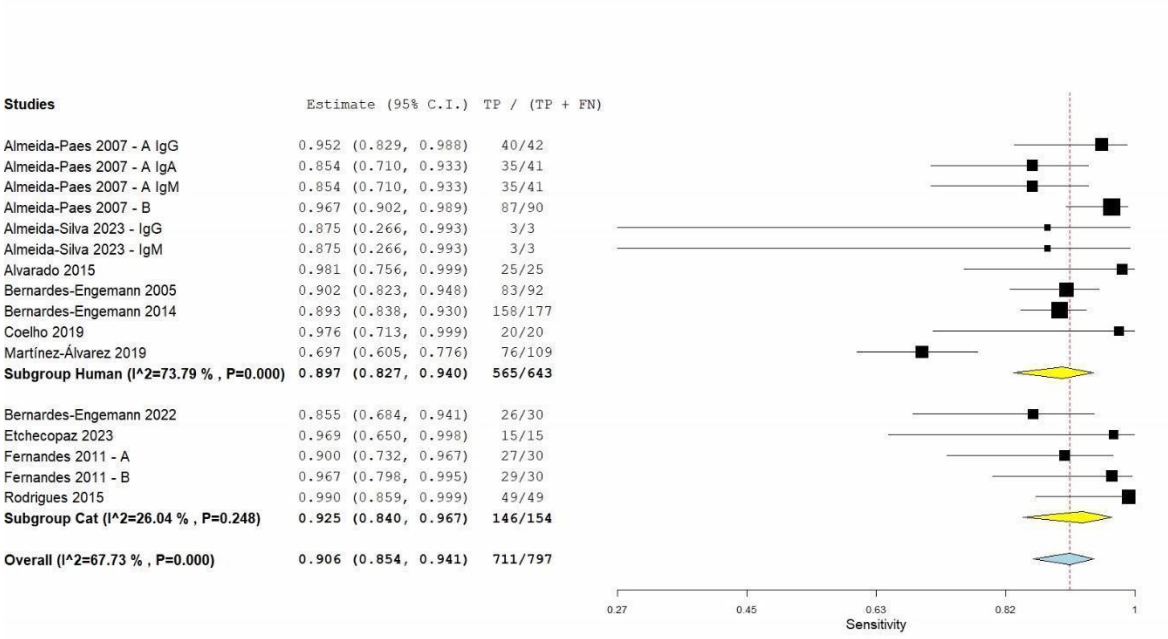
Supplemental Content

Table S1. Search Results

Database	Terms	Results
Pubmed	((Sporotrichosis[Title/Abstract]) OR (Sporothrix[Title/Abstract])) AND ((ELISA[Title/Abstract]) OR (Enzyme-Linked Immunosorbent Assay[Title/Abstract]))	36
Cochane	#1 ("sporotrichosis"):ti,ab,kw OR ("sporothrix"):ti,ab,kw (Word variations have been searched) 19 #2 ("ELISA"):ti,ab,kw OR ("enzyme linked immunosorbent assay"):ti,ab,kw (Word variations have been searched) 17305 #3 #1 AND #2 0	0
Web of Science	#1 (AB=(Sporotrichosis)) OR AB=(Sporothrix) #2 (AB=(ELISA)) OR AB=(Enzyme-Linked Immunosorbent Assay) #3 #1 AND #2	31
Scopus	((TITLE-ABS-KEY (sporotrichosis) OR TITLE-ABS-KEY (sporothrix))) AND ((TITLE-ABS-KEY (enzyme-linked AND immunosorbent AND assay) OR TITLE-ABS-KEY (elisa)))	108
EMBASE	#1 sporotrichosis:ab,ti,kw OR sporothrix:ab,ti,kw #2 'enzyme-linked immunosorbent assay':ab,ti,kw OR elisa:ab,ti,kw #3 #1 AND #2	53
LILACS	Sporotrichosis OR Sporothrix AND Enzyme-Linked Immunosorbent Assay OR elisa	8
Science Direct	(Sporotrichosis OR Sporothrix) AND (Enzyme-Linked Immunosorbent Assay OR elisa)	10
Periódicos Capes	Sporotrichosis OR Sporothrix AND Enzyme-Linked Immunosorbent Assay OR elisa	90
Total		**336**

31, January. 2024.

Figure S1. Sensitivity and specificity subgroup forest plot: Specie



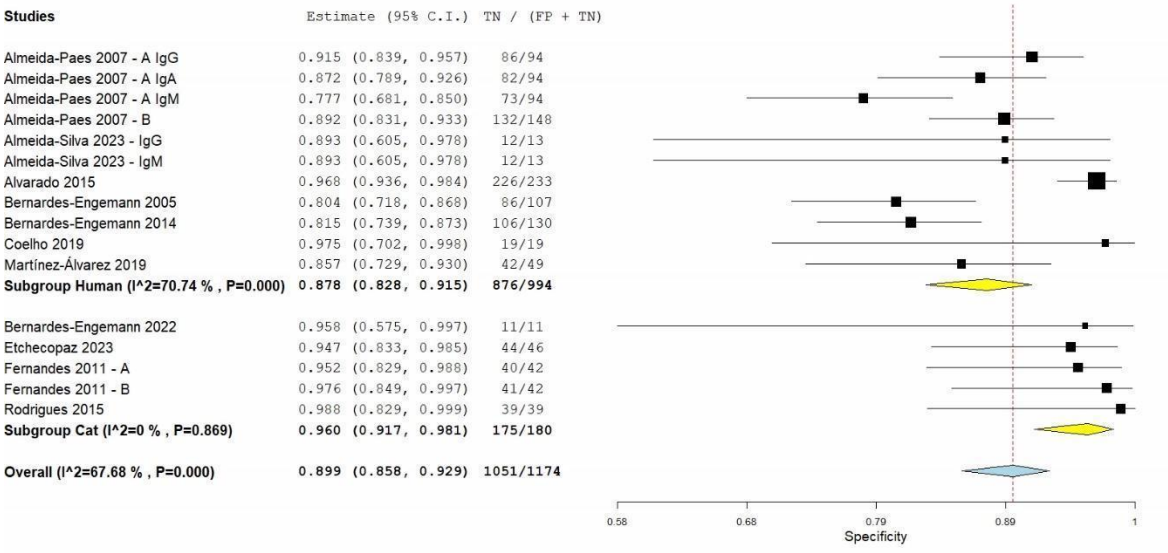


Figure S2. NLR and PLR forest plots

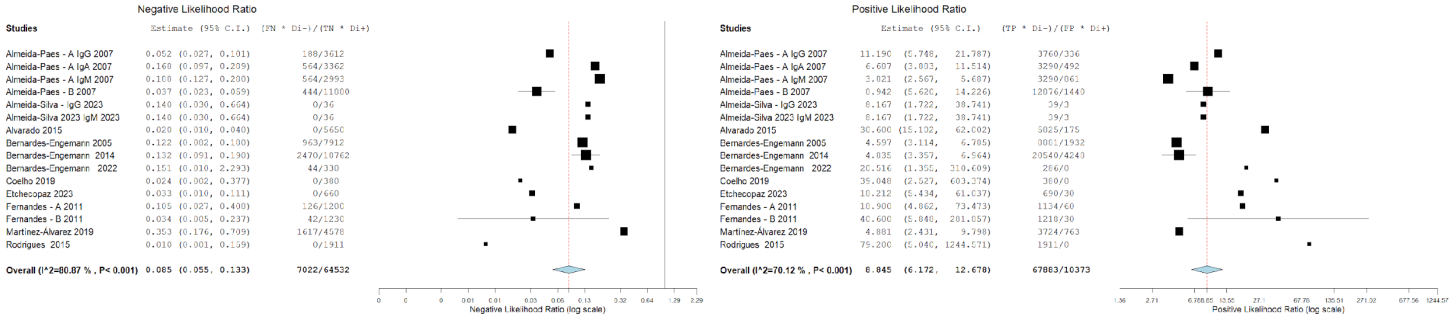


Figure S3. Diagnostic odds ratio subgroup forest plot: Specie

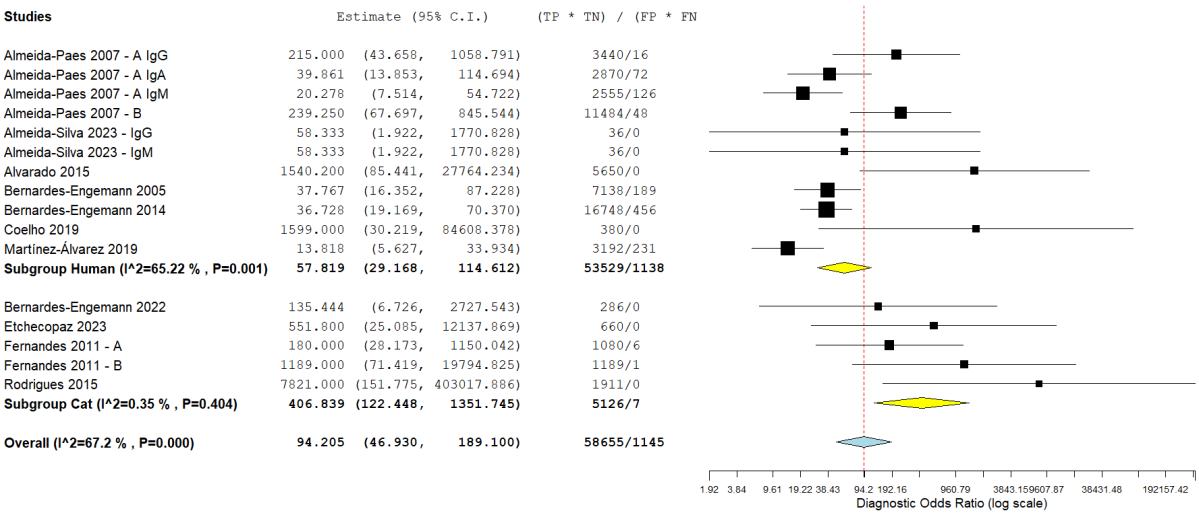


Figure S4. NLR and PLR subgroup forest plot: Specie

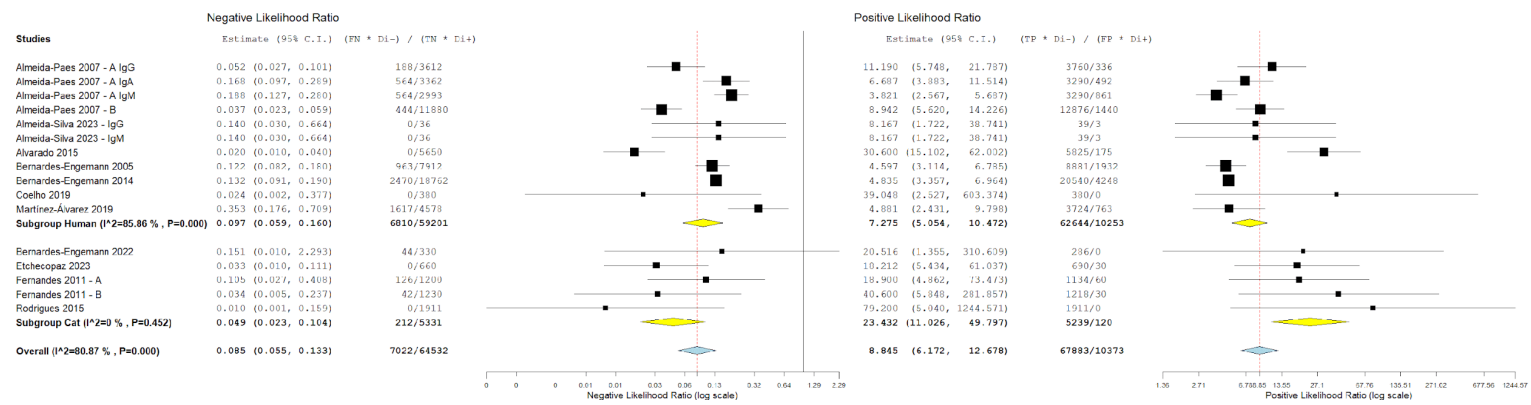
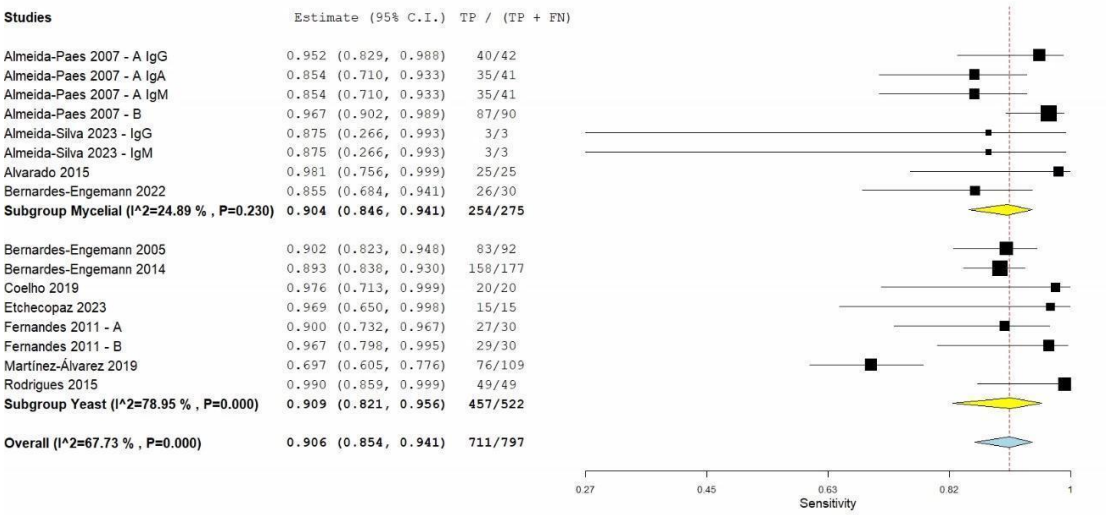


Figure S5. Sensitivity and specificity subgroup forest plot: Fungal form



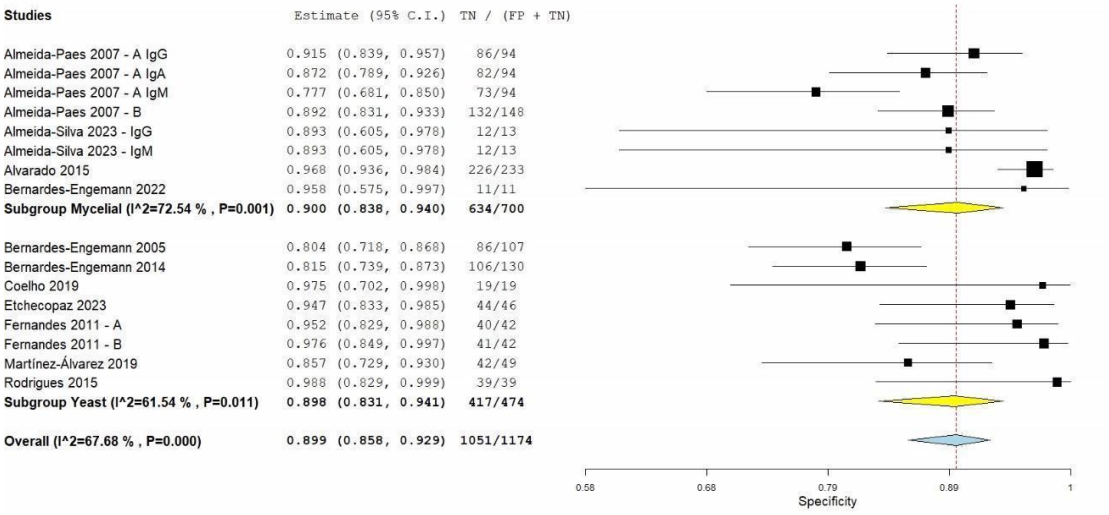


Figure S6. Diagnostic odds ratio subgroup forest plot: Fungal form

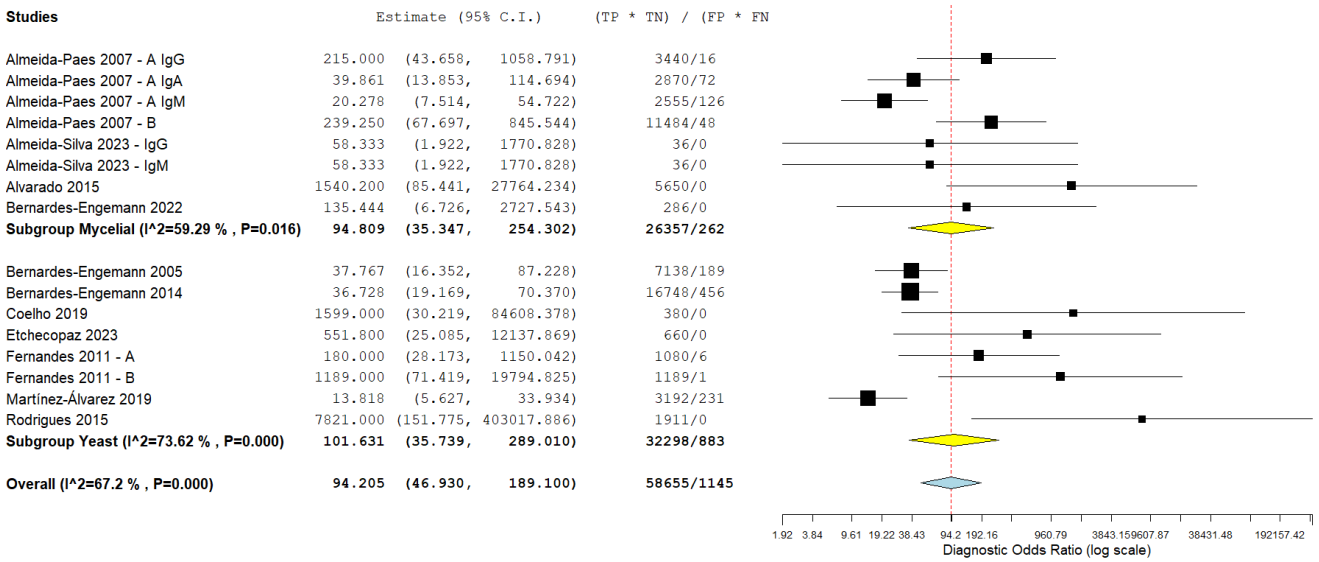


Figure S7. NLR and PLR subgroup forest plot: Fungal form

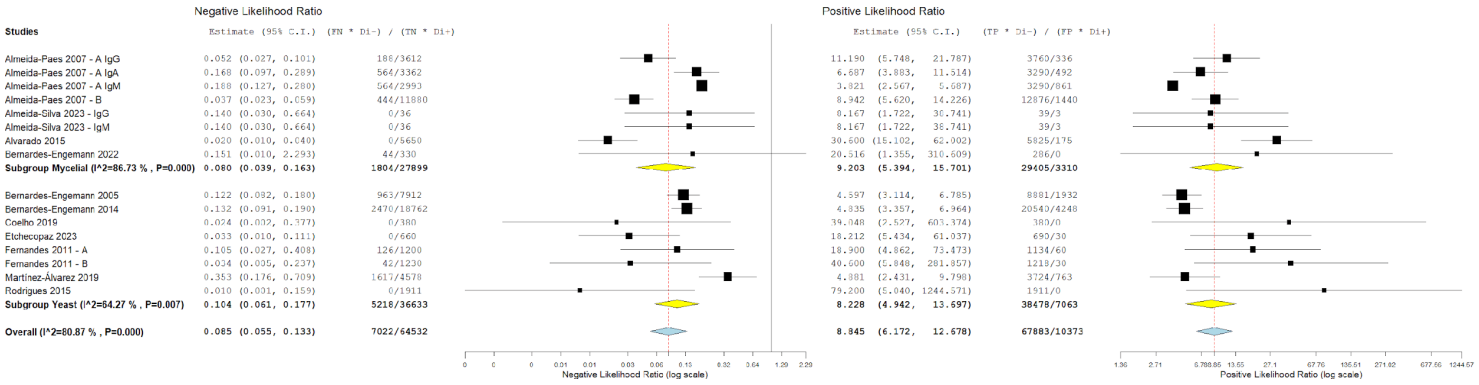
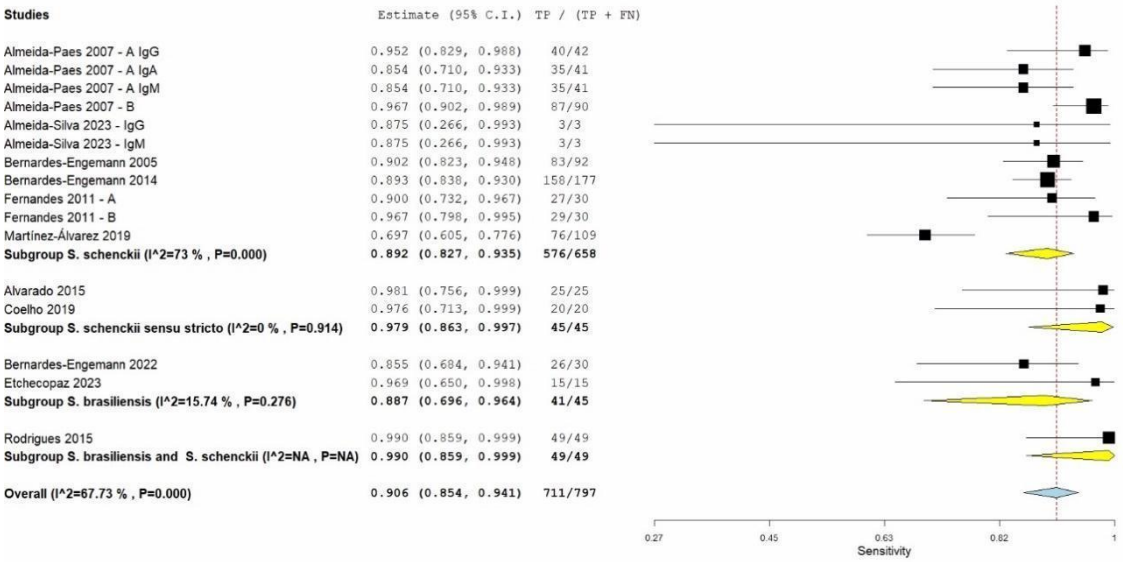


Figure S8. Sensitivity and specificity subgroup forest plot: Fungal specie



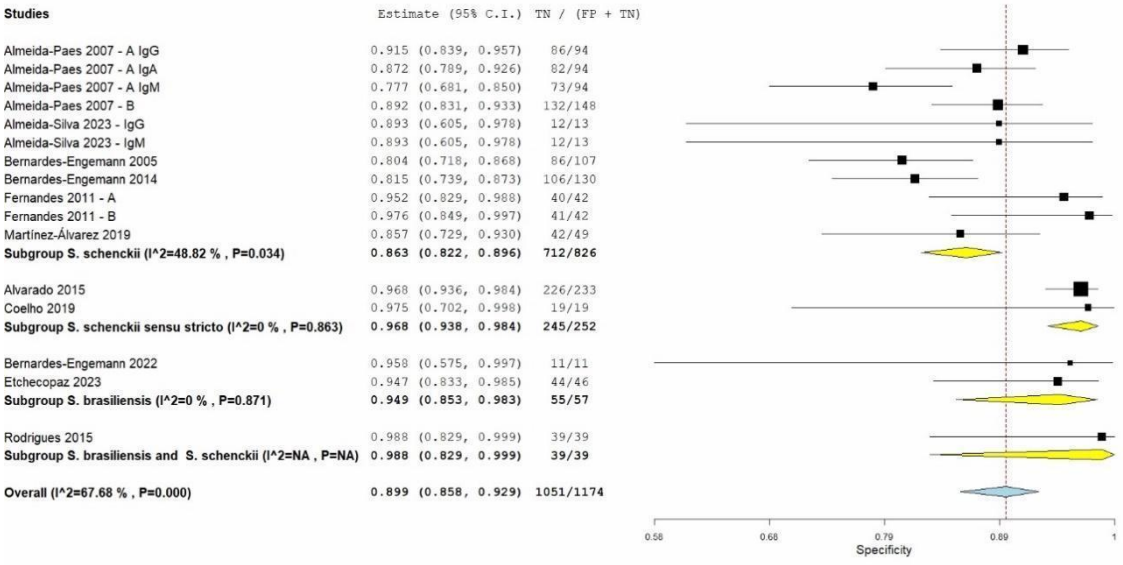


Figure S9. Diagnostic odds ratio subgroup forest plot: fungal specie

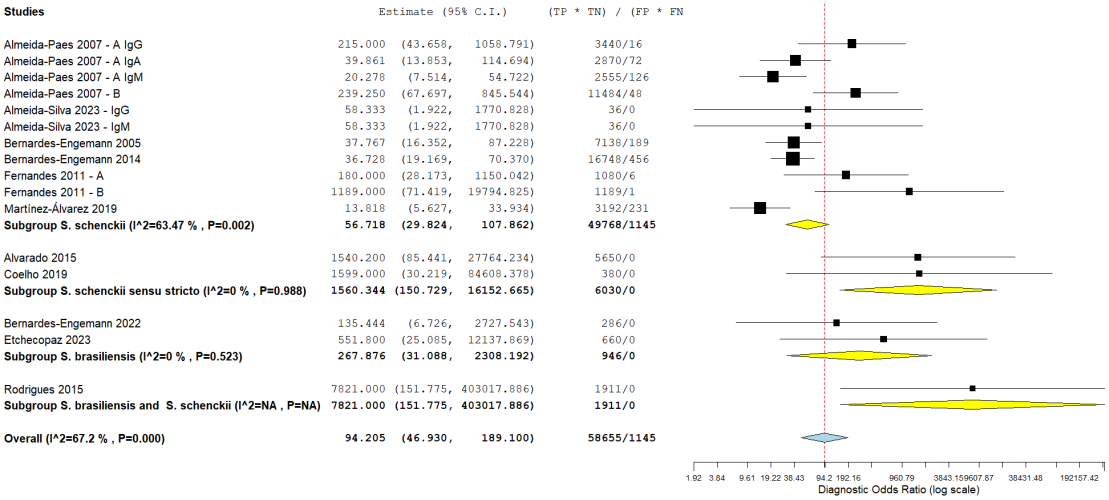


Figure S10. NLR and PLR subgroup forest plot: Fungal specie

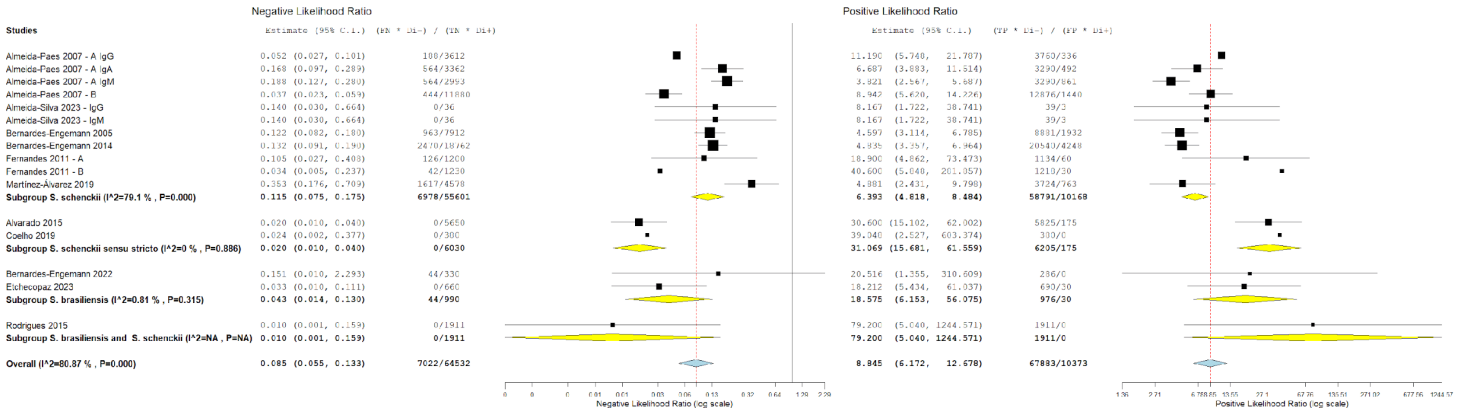


Figure S11. Diagnostic odds ratio subgroup forest plot: specie

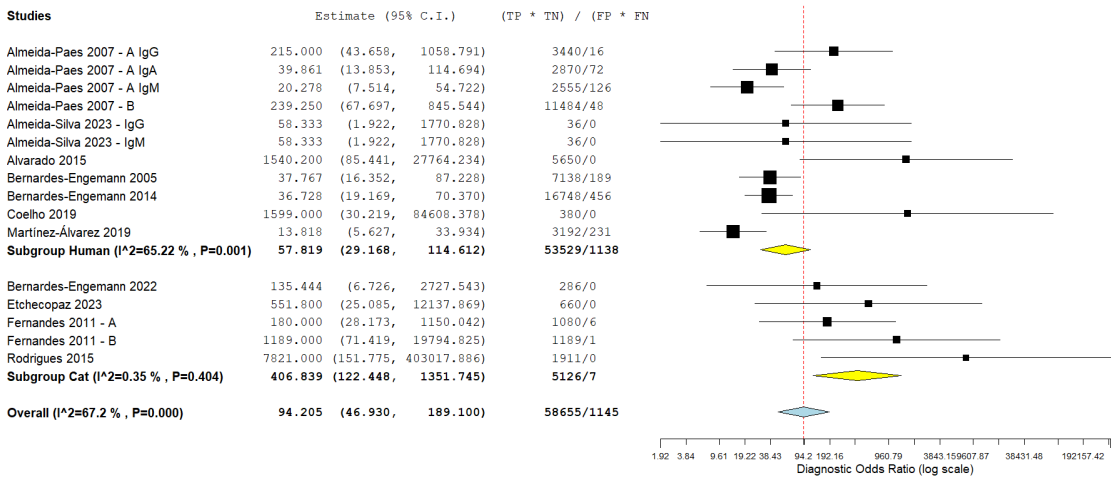


Figure S12. NLR and PLR subgroup forest plot: specie

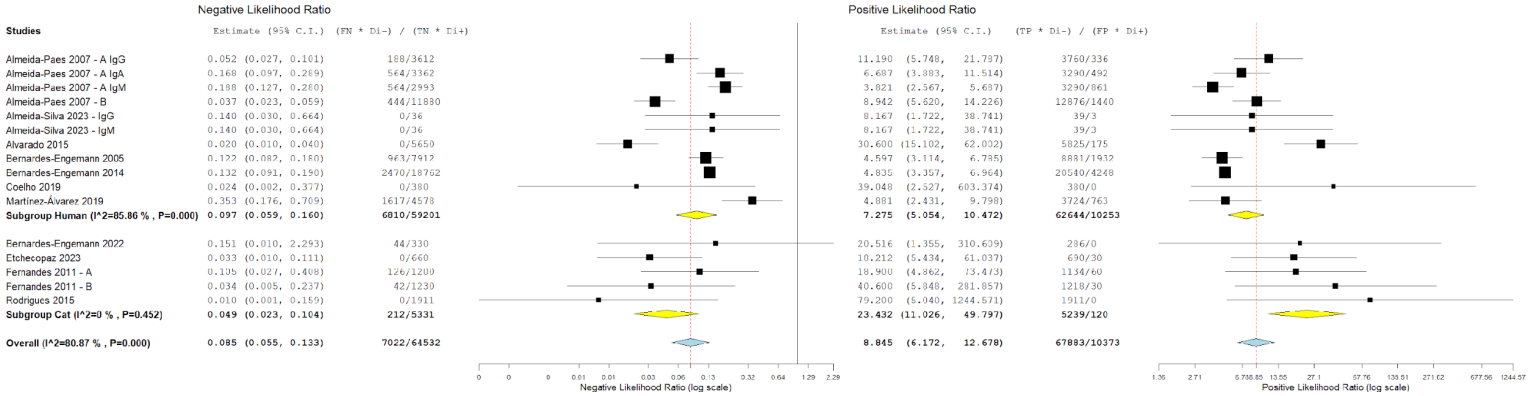
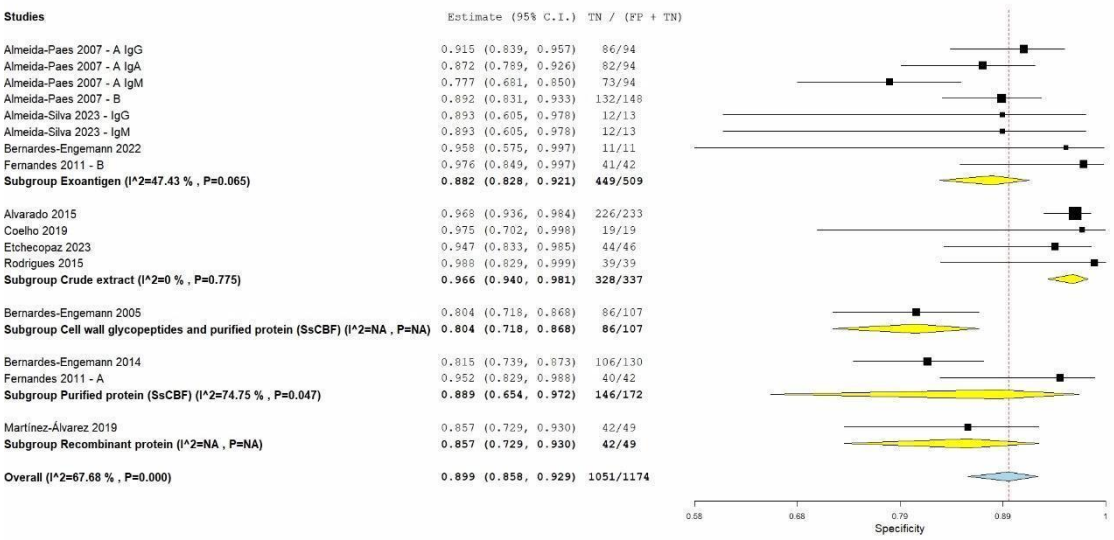


Figure S13. Sensitivity and specificity subgroup forest plot: type of antigen



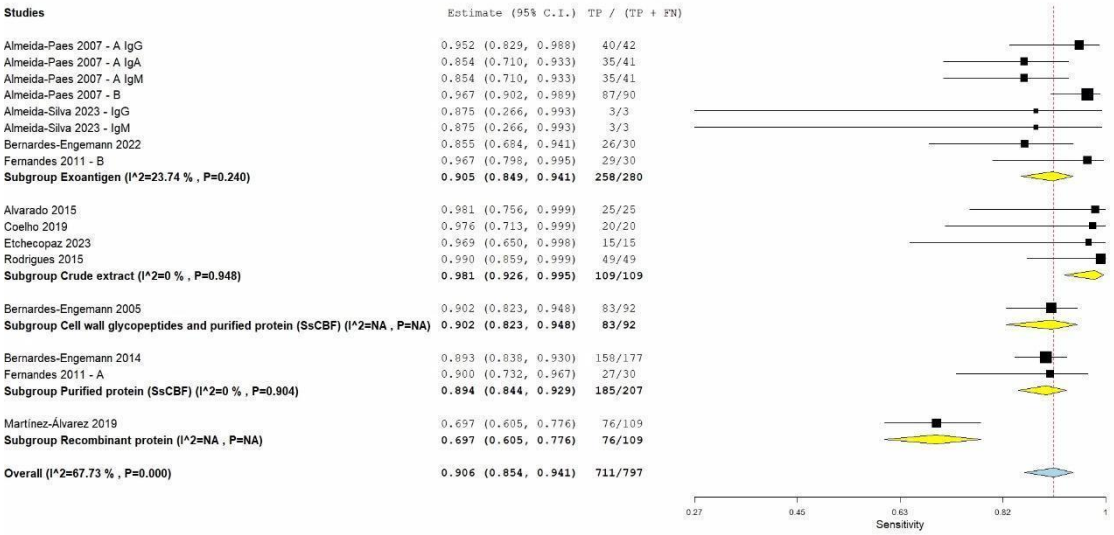


Figure S14. Diagnostic odds ratio subgroup forest plot: type of antigen

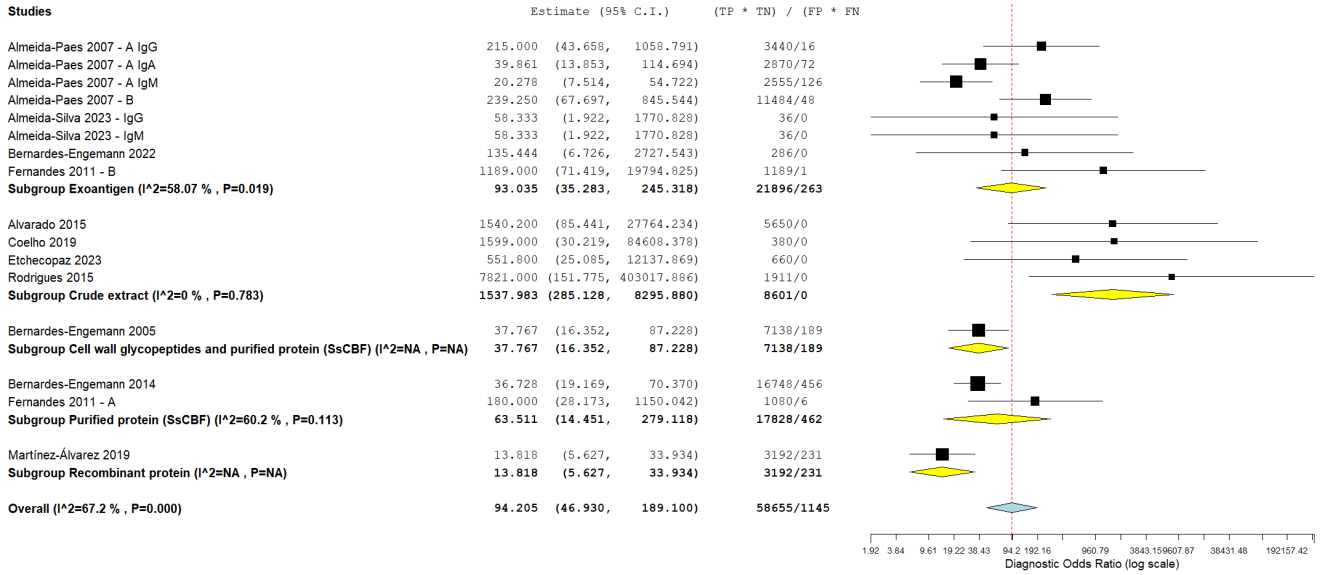


Figure S15. NLR and PLR subgroup forest plot: type of antigen

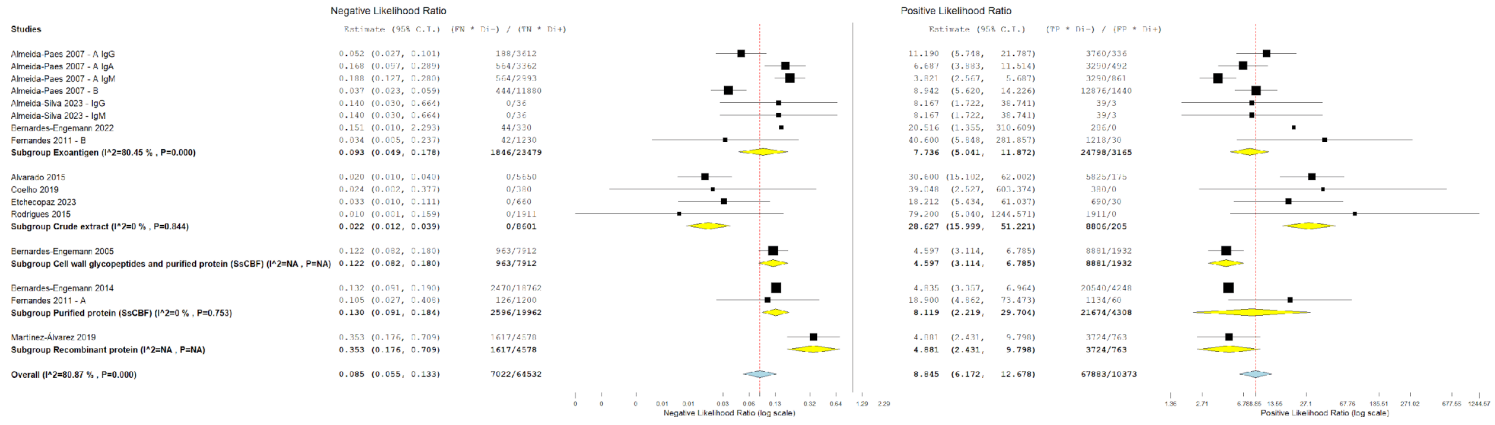


Figure S16. SROC plot: fungal form

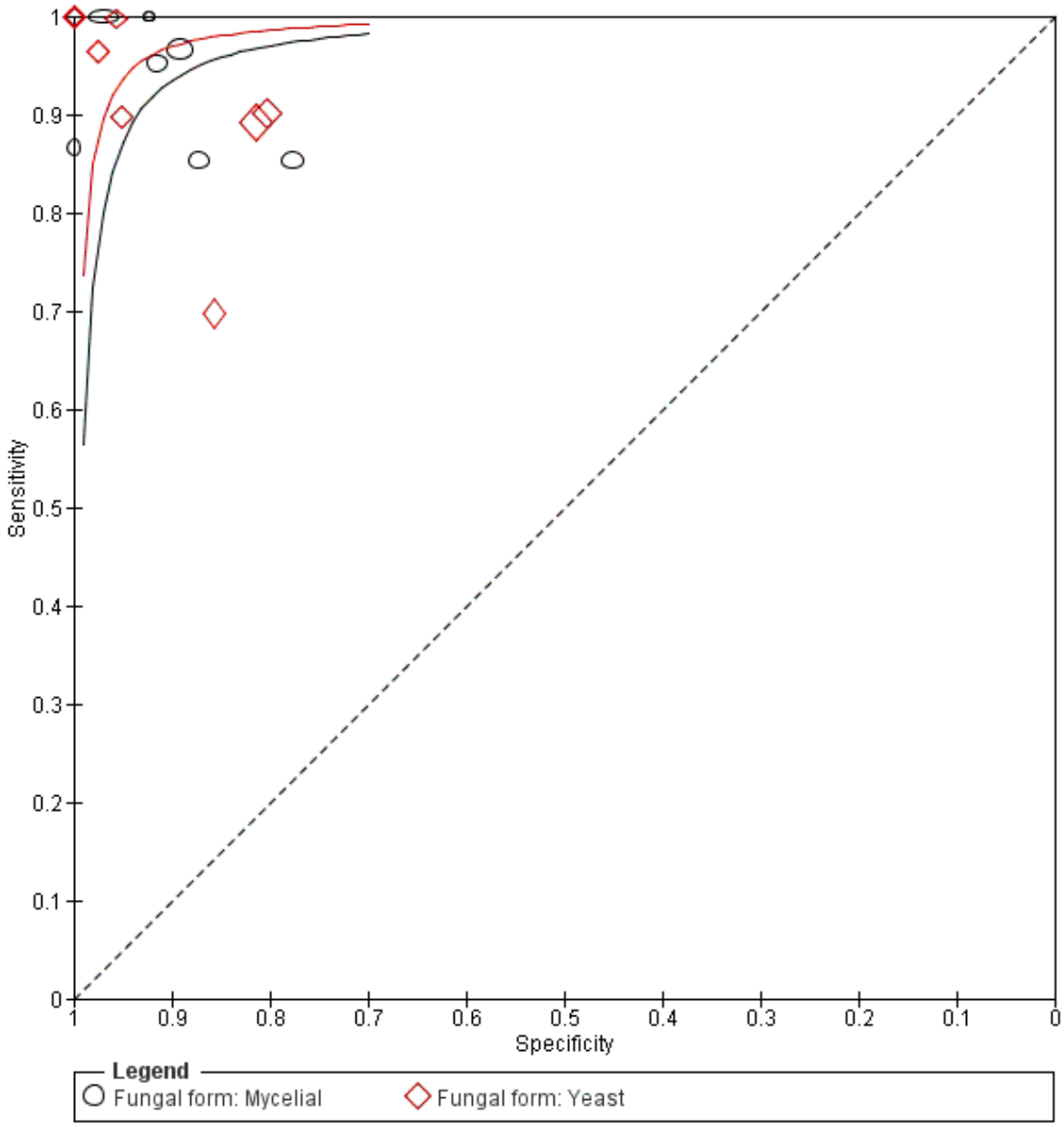


Figure S17. SROC plot: fungal specie

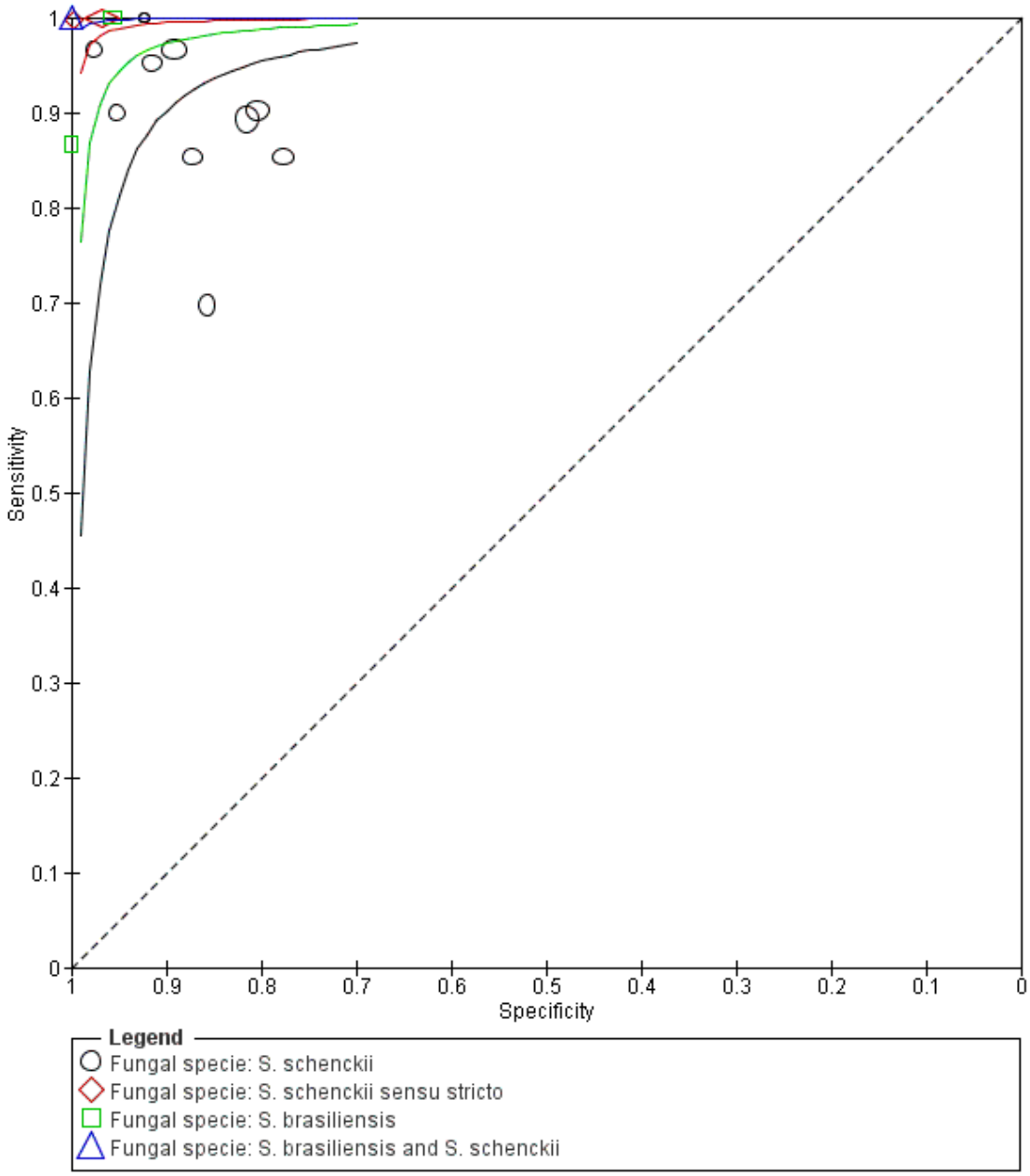


Figure S18. SROC plot: specie

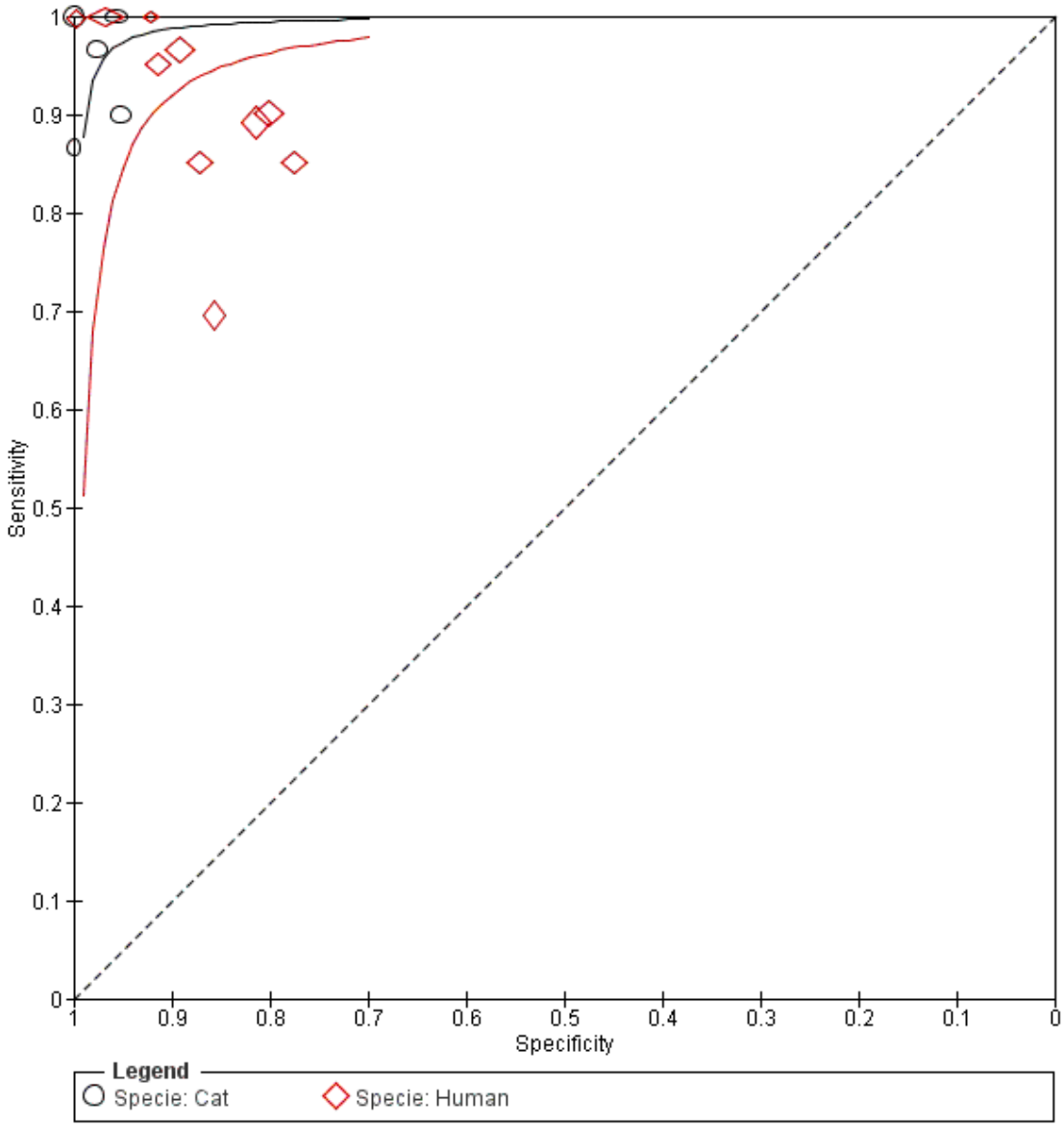
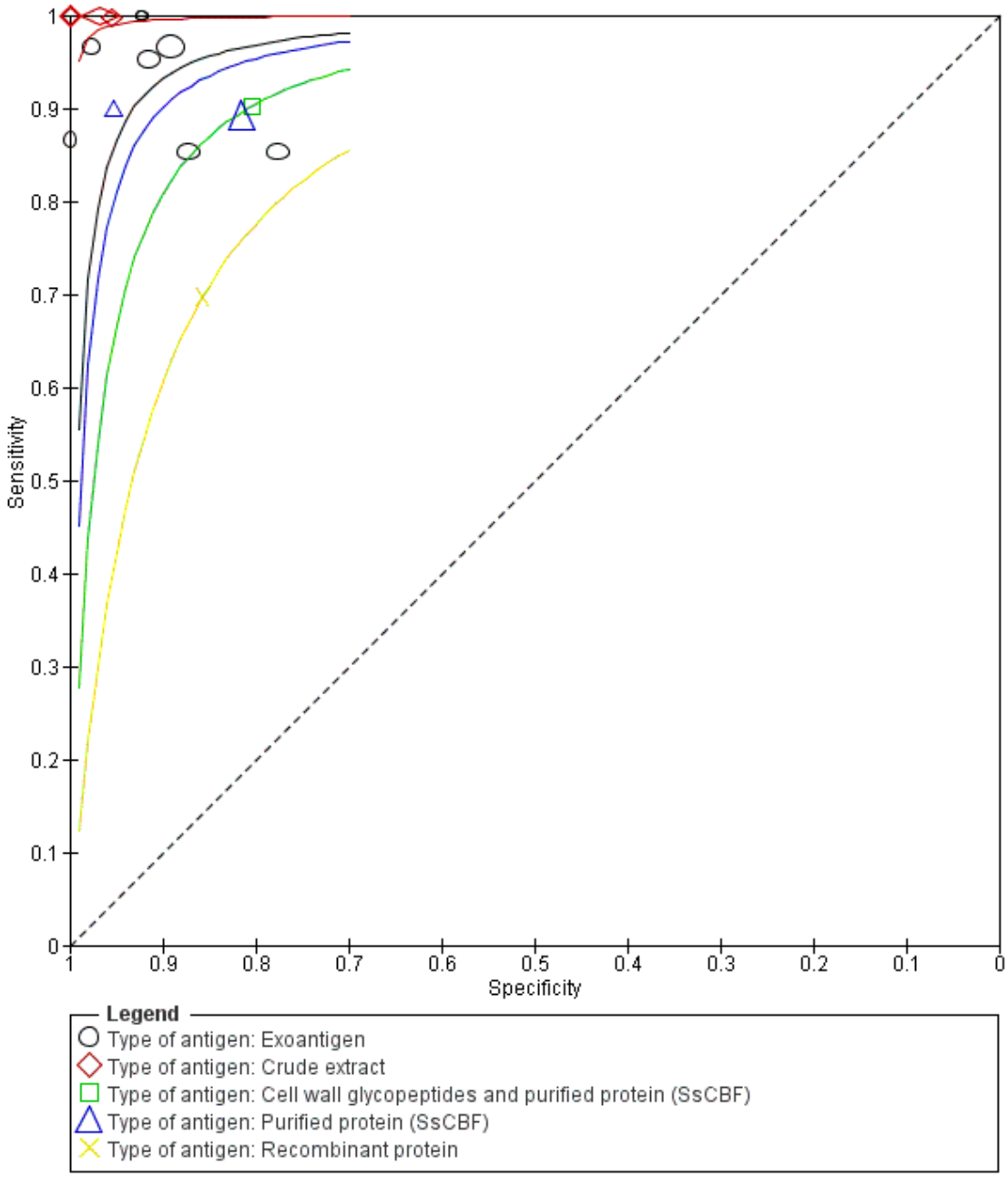


Figure S19. SROC plot: type of antigen



4 Considerações Finais

A partir da dissertação proposta, um ensaio de imunoadsorção enzimática (ELISA) utilizando uma proteína recombinante de *S. brasiliensis* para diagnóstico de esporotricose felina foi desenvolvido. O teste performou com 88% de sensibilidade e 100% de especificidade, sendo considerado satisfatório pelo planejamento do projeto realizado.

Ainda mais, por meio da revisão sistemática e meta-análise realizada com a mesma temática, foi possível esclarecer sobre a alta especificidade e sensibilidade dos estudos encontrados, seja em humanos e animais. Por fim, conclui-se que o ELISA é uma ferramenta diagnóstica eficiente que pode contribuir na melhora de políticas públicas e estratégias de vigilância sanitária no combate à doença.

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