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Desenvolvimento de ELISA indireto e teste imunocromatográfico com antígenos recombinantes TES30 e TES120 para o diagnóstico da toxocaríase

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Para meus pais, com carinho e gratidão.
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“A ciência nunca resolve um problema sem criar pelo menos outros dez”

George Bernard Shaw

Resumo

SANTOS, Lucas Moreira dos. **Desenvolvimento de ELISA indireto e teste imunocromatográfico com antígenos recombinantes TES30 e TES120 para o diagnóstico da toxocaríase.** 2019. 119f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A toxocaríase é uma doença negligenciada cosmopolita capaz de acometer inúmeros animais. Em humanos, a larva pode causar danos durante meses ou até anos, e dependendo da localização, esses danos podem ser irreversíveis. Seu difícil diagnóstico é um dos principais pontos de contribuição para seu carácter de doença negligenciada. Com uma ampla gama de sinais e sintomas não específicos, o diagnóstico é baseado em sinais clínicos, histórico do paciente, eosinofilia e pesquisa de anticorpos contra IgE total e anticorpos específicos. A pesquisa de anticorpos específicos baseia-se na produção nativa de antígenos de excreção e secreção do *Toxocara canis* (TES), de forma laboriosa, especializada, com amplo uso de insumos e tempo. Como alternativa, estudos têm avaliado se proteínas recombinantes teriam a capacidade de melhorar o atual panorama de diagnóstico da doença. Dessa forma, esta tese objetivou desenvolver e avaliar um teste imunoenzimático e imunocromatográfico com proteínas recombinantes de antígenos de excreção e secreção de *T. canis* (rTES-30 e rTES-120) para diagnóstico e estudos epidemiológicos da infecção por *T. canis*. As proteínas recombinantes expressas em *Escherichia coli* apresentaram, em testes imunoenzimáticos (ELISA), sensibilidades e especificidades (acima de 81%) em modelo experimental (camundongo), humanos, bovinos, ovinos e equinos. A sensibilidade, entre 56 a 69%, das proteínas expressas em *Pichia pastoris* não favorece seu uso no imunodiagnóstico da toxocaríase. Ademais, o protótipo de teste imunocromatográfico apresentou capacidade de diagnóstico em humanos com sensibilidade e especificidade, de 74 e 73%, respectivamente. Concluindo, o presente trabalho demonstrou avanço no diagnóstico de toxocaríase, apresentando um modelo funcional para diagnóstico imunoenzimático e imunocromatográfico.

Palavras-chave: *Toxocara* spp., Larva migrans visceral, Imunodiagnóstico, DNA recombinante, Teste rápido, ELISA.

Abstract

SANTOS, Lucas Moreira dos. **Development of indirect ELISA and immunochromatographic test with recombinant antigens TES30 and TES120 for diagnosis of toxocariasis.** 2019. 119f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Toxocariasis is a neglected cosmopolitan disease capable of affecting numerous animals. In humans, the larva can cause damage for months or even years, and depending on its location, such damage may be irreversible. Its difficult diagnosis is one of the main contributing points to its neglected disease character. With a wide range of non-specific signs and symptoms, diagnosis are based on clinical signs, patient history, eosinophilia, and screening for antibodies against total IgE and specific antibodies. The search for specific antibodies is based on the native production of *Toxocara canis* (TES) excretion and secretion antigens in a laborious, specialized manner, with extensive use of inputs and time. Alternatively, studies have evaluated whether recombinant proteins would have the ability to improve the current disease diagnosis landscape. Thus, this thesis aimed to develop and evaluate an immunoenzymatic and immunochromatographic test with recombinant proteins of *T. canis* excretion and secretion antigens (rTES-30 and rTES-120) for diagnosis and epidemiological studies of *T. canis* infection. The recombinant proteins expressed in Escherichia coli showed, in immunoassay tests (ELISA), sensitivities and specificities (above 81%) in experimental model (mouse), human, bovine, ovine and equine. The sensitivity, between 56 and 69%, of the proteins expressed in Pichia pastoris does not favor their use in toxocariasis immunodiagnosis. Moreover, the prototype immunochromatographic test showed diagnostic capacity in humans with sensitivity and specificity, of 74 and 73%, respectively. In conclusion, the present work demonstrated progress in the diagnosis of toxocariasis, presenting a functional model for immunoenzymatic and immunochromatographic diagnosis.

Keywords: *Toxocara* spp., Visceral larva migrans Immunodiagnosis, recombinant DNA, Rapid test, ELISA.

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1. INTRODUÇÃO GERAL

A toxocaríase humana é uma zoonose causada pelos nematódeos *Toxocara canis e Toxocara cati*, que habitam o intestino de caninos e felinos, respectivamente (Fischer, 2018). O infecção se dá, principalmente pela ingestão de ovos embrionados presentes em mãos sujas, hortaliças mal higienizadas, no pêlo de cães e no solo pela geofagia (Amaral et al., 2010; Despommier, 2003). Também foram relatados casos de toxocaríase decorrentes do consumo de carnes ou vísceras mal cozidas ou cruas contendo larvas infectantes de *T. canis* de hospedeiros paratênicos, como aves e bovinos (Choi et al., 2008; Morimatsu et al., 2006). Assim como ocorre em cães e gatos, a transmissão vertical também já foi relatada em humanos (Maffrand et al., 2006).

Em humanos, a toxocaríase ocorre, principalmente, devido à infecção por *T. canis*. As larvas invadem a parede do no intestino delgado e migram para outras regiões do corpo. Ocorrem três principais formas clínicas de toxocaríase: a síndrome da *larva migrans visceral* (LMV), caracterizada pela migração da larva para órgãos como fígado, encéfalo, linfonodos, pulmão, entre outros; a síndrome da *larva migrans ocular* (LMO), caracterizada pela migração da larva para os olhos; e a toxocaríase oculta (*covert toxocariasis*), designada dessa forma pelos sinais subclínicos. Apesar dessas classificações, os sintomas decorrentes da LMV e toxocaríase oculta não diferem demasiadamente, sendo, inclusive, similares aos de outras doenças (Smith et al., 2009). Entretanto, a LMO apresenta importante morbidade e geralmente acomete crianças acima de seis anos de idade e adultos jovens, desencadeando perda parcial ou total da acuidade visual. A principal consequência é a formação de um granuloma característico que pode ser detectado no exame de fundo de olho (Despommier, 2003; Magnaval et al., 2001; Smith et al., 2009).

A dificuldade de distinguir as formas clínicas, com exceção à LMO, associada aos diferentes sítios que as larvas de *Toxocara* spp. podem se alojar no organismo humano dificultam o diagnóstico desta parasitose (Despommier, 2003; Magnaval et al., 2001; Smith et al., 2009). Desse modo, abordagens imunológicas têm recebido atenção para o diagnóstico específico da doença. Para o diagnóstico da infecção por *T. canis*, o método imunológico utiliza os antígenos excretório-secretórios das larvas (TES) em ensaio de imunoadsorção enzimática (ELISA), conhecido como ELISA-

TES. Entretanto, a produção do antígeno TES nativo é laboriosa, dispendiosa e necessita de pessoal treinado, uma vez que se trabalha com o parasito vivo (Mohamad et al., 2009; Peixoto et al., 2011), fatores que dificultam a implementação deste teste na rotina laboratorial. Em 2016, investigou-se a soroprevalência em agentes de saúde que atuavam n a produção de TES nativo, nenhuma associação positiva foi evidenciada, embora 13.8% dos trabalhadores não usassem luvas para o manuseio de ovos do parasito (Mattos et al., 2016). Além disso, o ELISA-TES tem apresentado resultados falso positivos em cachorros infectados por *Ancylostoma caninum* (Elsemore et al., 2017).

Neste contexto, o uso de proteínas recombinantes tem se apresentado como excelente alternativa, permitindo a fácil obtenção desses antígenos bem como o desenvolvimento de diagnóstico com alta especificidade e sensibilidade para toxocaríase, além da segurança laboratorial, uma vez que não é necessária a manipulação do parasito (Moreira et al., 2014). Empregando essa abordagem, o ELISA-TES com as proteínas recombinantes TES-30 e TES-120 (ELISA-rTES) permitiu a obtenção de testes com especificidade e sensibilidade superior a 90% (Mohamad et al., 2009; Norhaida et al., 2008).

Métodos rápidos de diagnóstico, tipo *point-of-care*, são ferramentas valiosas para incrementar o controle de doenças infecto-parasitárias. Neste contexto, testes imunológicos são mais rápidos na obtenção dos resultados e de maior facilidade de execução, facilitando ou possibilitando estudos de soroepidemiologia em populações situadas em regiões vulneráveis, a fim de incorporar medidas de prevenção, controle e tratamento eficazes (Kundu et al., 2018; Odaga et al., 2014).

Diante do exposto, o desenvolvimento de um ensaio imunoenzimático e um teste rápido imunocromatográfico com antígenos recombinantes de *Toxocara canis* é estratégico para o diagnóstico e a vigilância epidemiológica da toxocaríase, possibilitando aprofundar o conhecimento sobre as consequências resultantes da exposição ao parasito bem como o impacto na saúde pública.

2. REVISÃO BIBLIOGRÁFICA

2.1 *Toxocara*: Os agentes causais da toxocaríase

O gênero *Toxocara* constituí nematódeos gastrointestinais e potenciais causadores de uma zoonose, chamada toxocaríase, em humanos. Larvas desse gênero possuem uma fase migratória tecidual que se estende durante meses ou anos, atingindo sistemas no corpo do hospedeiro (Holland and Smith, 2006).

A primeira infecção por *Toxocara* spp. relatada na literatura aconteceu em 1950, por Wilder (Wilder, 1950), quando descreve um nematódeo desconhecido que infectava a cavidade ocular (LMO) de 24 de um total de 46 crianças em seu estudo sobre retino blastomas; a larva nessa época era descrita como um ancilostomídeo. Posteriormente, Beaver e colaboradores descreveram os primeiros casos de LMV em crianças, que apresentavam eosinofilia e enfermidades multisistêmicas crônicas e severas; o estudo de Beaver foi também o primeiro a, corretamente, descrever os agentes causais como ascarídeos nematódeos *Toxocara canis* e *T. cati*, parasitos cosmopolitas de cães e gatos, respectivamente (Beaver et al., 1952).

Atualmente, conhece-se uma variedade de espécies do gênero *Toxocara*. Fora as já relatadas que infectam humanos: *T. canis*, *T. cati* e *T. malaysiensis* (descoberta em gatos domésticos na Malásia); tem sido estudado as espécies *T. vitulorum* (bovinos), *T. apodemi* (camundongo), *T. lyncus* (caracal), *T. mackerrasae* (roedores), *T. paradoxura* (viverridae), *T. sprenti* (viverridae), *T. vajrasthirae* (mustelídeos), *T. tanuki* (guaxinim) e *T. pteropodis* (morcegos); que ainda não se tem conhecimento se possuem capacidade de infectar humanos (Alexander et al., 2018; Asakawa et al., 1994; Despommier, 2003; El-Ashram et al., 2019; Gasser et al., 2016; Warren, 1970).

2.2 Genética e Proteômica de *Toxocara canis*

O genoma de *T. canis* é composto de 18 cromossomos nas células germinativas, que originam 20178 genes. Contudo, devido a sua semelhança com outras espécies parasitárias como: *Caenorhabditis elegan*; *Trichinella spiralis*; *Brugia malayi*; e *Ascaris suum*; apenas 6155 genes são únicos ao *T. canis*. Embora nenhuma comparação completa genômica foi realizada, é esperado que *T. canis, T. cati* e *T. vitulorum* apresentem alta homologia (Holland and Smith, 2006; Kong et al., 2016; Sperotto et al., 2017).

Como o nematódeo foi sequenciado apenas em 2016, e até o momento não foi completamente anotado, poucos estudos de proteômica estão disponíveis para elucidação do parasito (Kong et al., 2016). Estudos de proteômica têm focado suas atenções sobre os produtos que o parasito secreta (TES) e que são de importância para o diagnóstico e imunidade ao parasito (Holland and Smith, 2006; Sperotto et al., 2017).

A família de proteínas TES são compostas de 870 distintas proteínas, das quais 732 tem função desconhecida. Dentre as que têm função conhecida e exclusivas ao *Toxocara* spp., as lecitinas tipo C (TES-32) e as mucinas (TES-120) estão sendo atualmente consideradas como alvos para diagnóstico (Mohamad et al., 2009; Sperotto et al., 2017). Estas duas proteínas, TES-32 e TES-120, foram caracterizadas funcionalmente como proteínas relacionadas a formação da cutícula externa do parasito e sinalização neuronal (Zhu et al., 2015).

A proteína TES-32 é a proteína mais abundante do complexo TES, sendo caracterizada como uma proteína glicolisada de 32 kDa constituinte da cutícula externa do parasito, e com um domínio carboidrato muito semelhante ao de células mamíferas, o que a confere resistência ao sistema imune do hospedeiro através da competição das lectinas do hospedeiros, diminuindo a adesão e sinalização das células inflamatórias (Holland and Smith, 2006; Sperotto et al., 2017). A TES-120 atua de forma diferente aumentando a defesa imune do parasito contra o sistema imune celular do hospedeiro, através da sua secreção na parte externa da cutícula do parasito que competem com as cadeias glicêmicas na célula do hospedeiro e bloqueiam a leucocitose nos tecidos invadidos, permitindo que a infecção se permaneça por anos (Holland and Smith, 2006; Sperotto et al., 2017).

Fora essas duas importantes proteínas, Sperotto e colaboradores evidenciaram uma outra abundante proteína do complexo, a TES-26, contudo esta proteína apresenta alta divergência nos genes que a geram nos bancos de dados (Uniprot), indicando que esta proteína possivelmente está submetida a mutações rotineiramente (Sperotto et al., 2017). Em alguns casos de infecções, a larva também produz outras proteínas TES, dependendo da necessidade de ligantes teciduais, como é o caso da TES-70, que também é uma lecitina tipo C, mas correlacionada a servir de ligante mediada por cálcio para a superfície de células renais (Loukas et al., 2000).

2.3 Formas clínicas da toxocaríase

Com a migração das larvas para os diferentes sistemas, a patologia do parasito pode variar conforme sua localização nos tecidos do hospedeiro. Existem quatro síndromes causadas por parasitos do gênero *Toxocara*: a LMV, a LMO, a toxocaríase oculta (*covert*) e a neurotoxocaríase (Deshayes et al., 2016; Despommier, 2003; Magnaval et al., 2001; Smith et al., 2009).

Na LMV, os tecidos mais atingidos são hepáticos e pulmonares. Desta forma o paciente pode apresentar dores abdominais, redução de apetite, febre, tosse, hepatomegalia e asma. Além disso, é característica a eosinofilia, leucocitose, aumento de IgE total e altos níveis de interferon gama no sangue (Magnaval et al., 2001). Esses quadros só são notados quando o parasito realiza a migração, por isso *larva migrans visceral*, pois uma vez que o parasito adquiri sua forma cística (fixando-se a um local), deixa de apresentar qualquer tipo de sintoma, podendo ser reativado ao longo dos anos e realizar novas migrações (Santos et al., 2017). Assim, a doença é entendida como crônica, e o monitoramento sorológico do paciente se torna uma alternativa importante (Pawlowski, 2001).

Um dos possíveis desenvolvimentos após o tratamento da LMV, devido à baixa eficácia deste se o diagnóstico for tardio (Wiśniewska-Ligier et al., 2012), é a mudança de característica da síndrome, tornando-se o que se conhece por toxocaríase oculta ou *covert toxocariasis*. Síndrome descrita por Taylor e colaboradores em 1987; nessa síndrome, o paciente apresenta sorologia positiva e regressão pós-tratamento dos sintomas, mas estes podem retornar de forma mais amena, como uma dor abdominal persistente, tosse ou dor de cabeça, e persistindo durante meses ou até anos (Taylor et al., 1987; Nathwani et al., 1992). Essa síndrome pode também se apresentar de forma isolada a LMV, apenas com sinais e sintomas inespecíficos, dificultando o diagnóstico (Taylor et al., 1987; Nathwani et al., 1992).

Na LMO, o parasito tem tropismo para invadir as estruturas do olho, sendo o granuloma a lesão mais frequente. A consequência mais comum de um diagnóstico de granuloma é a fibrose, que pode evoluir, conforme a relação do sistema imune do hospedeiro e o parasito, até comprometer irreversivelmente a função do olho do paciente (Ahn et al., 2014). Diferentemente da LMV, a infecção ocular pode não gera a eosinofilia. Um estudo realizado em 2017 investigou 250 casos de suspeitas de LMO e foi encontrado soropositividade de anticorpos contra toxocaríase em 62% dos

pacientes, sendo associado como fator de risco a vitreite (p = 0,003) (Iddawela et al., 2017).

Além das patologias hepáticas, respiratórias e oculares, estudos têm apontado uma nova preocupação relacionada ao parasito: a neurotoxocaríase, associada a eosinofilia e meningite, meningoencefalite e encefalite, com possibilidade de alteração neurológica, redução motora e de excitabilidade (Janecek et al., 2017). Devido à migração dos parasitos, há a possibilidade de infecção no sistema nervoso, no qual biopsia não é possível de ser realizada. Além disso, a similaridade de sintomas semelhantes a doenças neurodegenerativas pode ocasionar uma falha de diagnóstico, impulsionando um tratamento errôneo e, consequentemente, agravação do quadro clínico (Abir et al., 2017).

2.4 Epidemiologia, prevenção e tratamento da toxocaríase

Com alta prevalência e falta de estudos em relação aos impactos na saúde humana, a toxocaríase é considerada uma das cinco parasitoses negligenciadas mais importantes, especialmente na América Latina, local onde o parasito encontra condições ambientais adequadas para seu desenvolvimento (Hotez and Wilkins, 2009). No Brasil, a toxocaríase também é uma doença negligenciada e subdiagnosticada nos serviços de saúde (Paludo et al., 2007), apesar de estudos sorológicos revelarem importante exposição de crianças ao *Toxocara* spp., com variação na taxa de prevalência de 28,8% a 54,8% (Alderete et al., 2003; Figueiredo et al., 2005; Paludo et al., 2007). As crianças estão expostas frequentemente ao risco de infecção por *Toxocara* spp. por terem estreito contato com solo contaminado (Figueiredo et al., 2005; Manini et al., 2012), cães jovens parasitados, pelos contaminados com ovos embrionados (Amaral et al., 2010; Carvalho and Rocha, 2011), caixas de areia de áreas de recreação infantil (Despommier, 2003) e contato com cães e gatos (Schoenardie et al., 2013).

Pelo perfil cosmopolita do parasito e a associação de uma zoonose relacionada a inúmeras espécies de hospedeiros, a prevenção contra a toxocaríase é enviesada para um perfil levemente reducionista de incidência (Alexander et al., 2018; Asakawa et al., 1994; Despommier, 2003; El-Ashram et al., 2019; Gasser et al., 2016; Warren, 1970). As técnicas de prevenção atualmente aplicadas estão vinculadas a evitar a indiscriminada deposição de fezes de cães e gatos em áreas públicas e tratamento

rotineiro com anti-helmínticos (Despommier, 2003). Deve-se ressaltar que devido à pouca compreensão de como o sistema imune atua sobre os estágios larvais do parasito nos sistemas do hospedeiro, nenhuma metodologia vacinal foi relatada na literatura (Holland and Smith, 2006).

Com uma prevenção limitada a medidas paliativas, faz-se importante duas etapas para o combate a negligência do parasito: o diagnóstico e o tratamento. No quesito tratamento, embora existam múltiplas síndromes associada a infecção por *Toxocara* spp., as alternativas aos tratamentos são limitadas, devido à necessidade dos medicamentos atingirem larvas encistadas em diversos tecidos e a dificuldade de verificar a eficácia das drogas nos pacientes (Ma et al., 2018).

Independente das dificuldades do tratamento, é recomendado albendazol e mebendazol em casos agudos de toxocaríase, prevenindo da larva atingir, através da migração, formas clínicas de neurotoxocaríase e LMO (Ahn et al., 2014; Janecek et al., 2017; Ma et al., 2018). Em casos já instalados de LMO, é necessário, além de anti-helmínticos, o uso de corticoides para diminuir a reação alérgica e prolongamento da fibrose, e, em casos extremos, remoção cirúrgica (Ma et al., 2018). Em casos já instalados de neurotoxocaríase, a única opção existente é o uso de albendazol, devido a penetração da barreira hemato-encefálica (Ma et al., 2018).

Entretanto, as eficácias dos atuais tratamentos são limitadas, conforme demonstrado na Tabela 1. Fora os sucessos em camundongos, as pesquisas em seres humanos, demonstram apenas em torno de 50% de sucesso no tratamento, com redução nos sintomas (Chen et al., 2018; Ma et al., 2018), sendo que pacientes com um diagnóstico mais precoce possuem chances maiores de sucesso (Chen et al., 2018).

Tabela 1. Tratamento de toxocaríase: eficácia das drogas anti-helmínticas. Fonte: Adaptado de Lescano et al., 2005; Ma et al., 2018.

Fármaco	**Eficácia**
Albendazol	Redução de 47% dos sintomas clínicos em pessoas infectadas
Dietilcarbamazina	Redução de 70% dos sintomas clínicos em pessoas infectadas

Ivermectina	Redução de 83,5% do número de larvas de camundongos infectados
Polietileno albendazol conjugado com glicol	79% de eliminação de larvas de encéfalo de camundongos infectados
Fenbendazol encapsulado em lipossomas estabilizado com PEG	90% de larvas eliminadas dos músculos e 66% dos encéfalo dos ratos infectados
Albendazol encapsulado em quitosana	100% de larvas eliminadas do encéfalo e aumento da eliminação do fígado e pulmões de camundongos infectados

Nota-se que todos os fármacos anti-helmínticas citadas no tratamento da toxocaríase, apresentam inúmeras reações adversas de tratamento, que varia de cinco dias a quatro semanas, dependendo dos sintomas e provável localização larval (Chen et al., 2018; Fan et al., 2015). Desta forma, fica evidente que possa ocorrer desistência do tratamento, principalmente por ser uma doença que afeta crianças e ter sintomas iniciais mais brando em comparação com algumas reações adversas medicamentosas (Alderete et al., 2003; Chen et al., 2018; Muñoz-Guzmán et al., 2010), sendo assim, o diagnóstico precoce primordial no combate à doença.

2.5 Diagnóstico da toxocaríase

Devido a multiplicidade dos órgãos afetados pelo parasito e seu carácter zoonótico cosmopolita, o diagnóstico da toxocaríase enfrenta grandes desafios, sendo um dos principais motivos da negligência da doença. O diagnóstico é baseia-se em sinais clínicos, epidemiológicossorologia, eosinofilia e pesquisa de IgE total (Fillaux and Magnaval, 2013).

Para um diagnóstico mais fidedigno, ambos exames devem sempre ser acompanhados de um histórico do paciente com fatores de risco predisponentes da doença: contato com cães (principalmente filhotes); uso de parques públicos urbanos;

alotriofagia; domicílio em região de baixa renda com ausência de saneamento básico; e baixo nível educational (Berrett et al., 2017; Despommier, 2003; Romero Núñez et al., 2013).

2.5.1 **Diagnóstico clínico da toxocaríase**

Os exames clínicos são pouco utilizados para confirmação da doença, devido a brandicidade dos sintomas; contudo, quando o paciente está na síndrome LMO, apresentando perda de visão unilateral e granulomas, é possível a suspeita da doença através da visualização da lesão na cavidade ocular em um exame de fundo de olho (Ahn et al., 2014; Despommier, 2003; Fonseca et al., 2019). Já a síndrome LMV é mais difícil de se diagnosticar clinicamente, sinais clínicos como hepatomegalia, febre e evidência de doença multisistêmica é um forte indicador para a investigação de toxocaríase através de exames laboratoriais (Despommier, 2003).

A neurotoxocaríase apresenta sintomas como meningite, encefalite e mielite; podendo-se ser confundidos com sintomas relacionados a demência e problemas com memória (Deshayes et al., 2016; Fan et al., 2015; Fillaux and Magnaval, 2013; Janecek et al., 2017; Sánchez et al., 2018). A toxocaríase oculta apresenta sintomas inespecíficos para suspeita clínica, tendo o mais prevalente sintoma uma dor abdominal recorrente (Nathwani et al., 1992; Taylor et al., 1987).

2.5.2 **Diagnóstico laboratorial da toxocaríase**

O diagnóstico laboratorial é o diagnóstico definitivo para toxocaríase, podendo ser caracterizado como direto, através de biópsias, e indireto, através da sorologia do paciente. Contudo, deve-se fazer menção de um exame laboratorial não-específico para toxocaríase que auxilia na confirmação da doença: o hemograma.

A contagem de células sanguíneas ou hemograma é útil principalmente devido a eosinofilia presente na síndrome LMV: sendo comum o paciente apresentar um aumento massivo de eosinófilos (em torno de 10000 células/mm³). Contudo, essa eosinofilia apresenta-se branda (1500 células/mm³) na toxocaríase oculta ou *covert* e desaparece na LMO, não sendo comum nessa síndrome. Devido ao aumento do número dos eosinófilos em algumas síndromes dessa patologia, também pode ocorrer o aumento de anticorpos do tipo IgE (Fillaux and Magnaval, 2013).

Pelo fato de que exames diretos necessitam de métodos invasivos, inviável na maioria dos pacientes, a forma de diagnóstico mais aplicável será o diagnóstico indireto ou sorológico (Fillaux and Magnaval, 2013).

O imunodiagnóstico da toxocaríase começou a ser desenvolvido logo após a identificação do parasito em 1950, sendo o uso de extrato de *Ascaris lumbricoides* ou *A. suum* publicado em 1958 capaz de causar aglutinação em contato com soros de pacientes infectados (Kagan, 1958). Como já se era esperado, um teste de hemaglutinação com extrato de outro helminto da mesma família gerava problemas de especificidade (Fillaux and Magnaval, 2013). Entretanto, mesmo utilizando extratos de *T. canis,* a reação cruzada ainda se mantinha (Fernando et al., 1970).

Em 1973, Niel e seus colaboradores demonstraram em imunoeletroforese que alguns antígenos TES não reagiam com soros de pessoas infectadas por *Ascaris* spp., contudo a imunoprecipitação necessária conferia baixa sensibilidade ao modelo de diagnóstico proposto (Magnaval et al., 1986; Niel et al., 1973). Finalmente, em 1979, Savigny uniu a descoberta do ELISA com o uso de antígenos TES obtidos *in vitro* para desenvolver o teste imunológico que é utilizado até hoje, o ELISA-TES, aumentando a sensibilidade do modelo (de Savigny et al., 1979; Niel et al., 1973; Savigny, 1975).

O modelo proposto por Savigny de produção de antígenos TES é ainda amplamente utilizado; caracteriza-se por ser um método extremamente laborioso, o qual apenas um número pequeno de laboratório tem a *expertise* para sua produção. Descrevendo brevemente o processo, conforme demonstrado na Figura 5, as fêmeas *Toxocara* spp. são coletadas de cães jovens após o tratamento com um anti-helmíntico (1). As fêmeas são submetidas a uma histerectomia para coleta dos ovos (2). Os ovos coletados são distribuídos em tubos de vidro que são tampados com algodão e contêm uma solução de NaCl a 0,9% suplementada com formol a 1%, incubados a 28 ° C em banho-maria agitado por 30 a 35 dias (3). Periodicamente, o nível de embrionamento é avaliado por inspeção microscópica, e quando a taxa é de aproximadamente 70% (4), ocorre a remoção da membrana mamilonada com hipoclorito de sódio 5% (5) para permitir a eclosão. Após lavagem e centrifugação, a etapa de eclosão (6) é realizada no meio Roswell Park Memorial Institute 1640 (RPMI-1640) em 37 ° C, com agitação com esferas de vidro para completar o rompimento das camadas de ovos (6). As larvas são então separadas em um aparelho de Baermann (7), e novamente cultivadas em meio (8) em meio RPMI-1640 por 21 dias

para gerar os antígenos TES. O sobrenadante da cultura é então concentrado por ultrafiltração e dialisada para remover o meio residual que pode interferir com aplicações diagnósticas (9). A solução que contém o TES antigênicos é então concentrada por filtração (10) (Fillaux and Magnaval, 2013; Moreira et al., 2014).

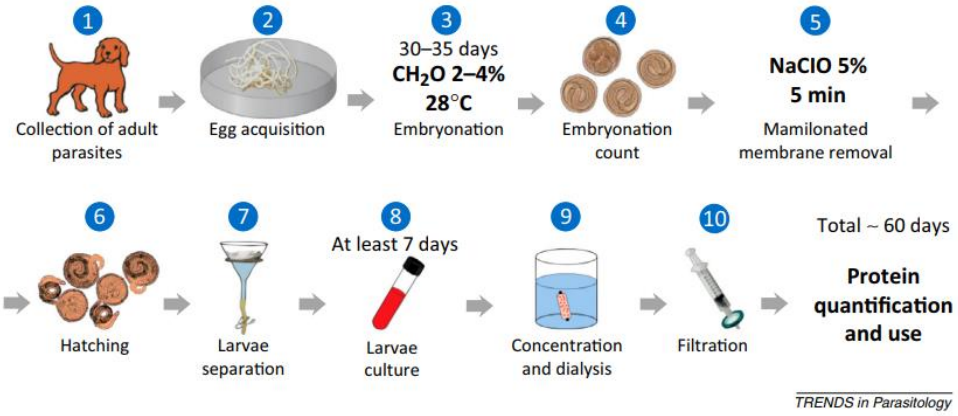

Figura 1. Produção de antígenos de excreção e secreção do *Toxocara canis* in vitro. Fonte: Moreira et al., 2014.

Além do laborioso processo de produção, a metodologia de Savigny traz inúmeros problemas de especificidade, devido a diferentes parasitos compartilharem alguns antígenos TES (Jin et al., 2015; Lescano et al., 2012; Nunes et al., 1999, 1997). Para contornar a inespecificidade com *Ascaris* spp., estudos têm realizado a adsorção do soro do paciente, previamente a realização do ELISA-TES, com um extrato de *Ascaris lumbricoides* (Souza et al., 2011), bem como a utilização de WB com TES nativo de forma confirmatória, no qual as proteínas com menor tamanho têm apresentado melhor especificidade (Morales et al., 2002; Qualizza et al., 2011; Roldán et al., 2015; Zibaei et al., 2013).

Com a descoberta de proteínas nativas de alta especificidade ao *Toxocara* spp., pesquisadores têm focado na produção de proteínas recombinantes em *Escherichia coli* e *Pichia pastoris* e aplicação em testes imunológicos (ELISA-rTES) (Fong and Lau, 2004; Mohamad et al., 2009). Metodologia que reduz o tempo de produção de 60 dias (de Savigny) para 5 dias; conforme pode ser observado na Figura 6, o gene da proteína de interesse é clonado em umas plasmídeo, esse plasmídeo é transformado em uma *E. coli* (1), o qual é cultivado para a expressão de proteína (2), após, é centrifugado e lisado a célula liberando a proteína para o meio (3), esse meio

é então purificado recuperando-se apenas a proteína de interesse (4) (Moreira et al., 2014).

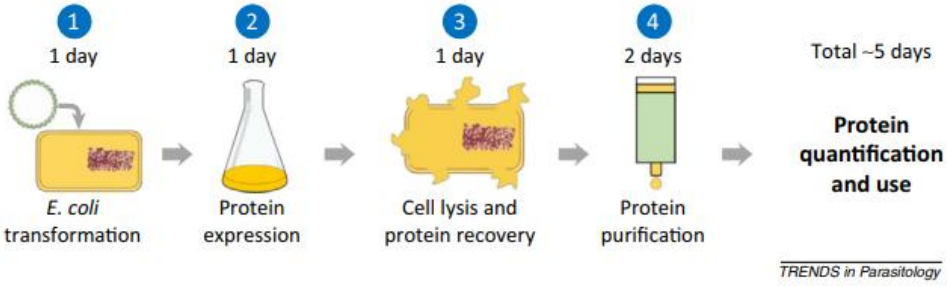

Figura 2. Produção de antígenos recombinantes. Fonte: Moreira et al., 2014.

Mohamad e colaboradores relataram uma sensibilidade de 80-100% e especificidade de 92-96,2%, dependendo da proteína recombinante a ser utilizada. Para obtenção dos altos níveis de sensibilidade e especificidade, Norhaida *et al*. (2008) e Mohamad *et al*. (2009) utilizaram a detecção a partir de IgG4. Os resultados obtidos com IgG4 contradiz estudo anterior publicados com TES nativo, no qual IgG2 e IgG3 apresentaram melhores sensibilidades e especificidades, respectivamente, em ELISA (Mohamad et al., 2009; Norhaida et al., 2008; Watthanakulpanich et al., 2008). A desvantagem do modelo de Mohamad e colaboradores, seria a necessidade de usar insumos de alto custo para cortar as caudas de solubilidade, aumentando demasiadamente o custo de produção (Mohamad et al., 2009).

Outra vantagem da utilização de antígenos recombinantes pertencentes à família de proteínas derivadas do TES está na sua reatividade com a espécie *T. cati*. Em estudo analisando se rTES-120 de *T. cati* (apresenta 84% de identidade com rTES-120 de *T. canis*) teria alguma capacidade de diagnóstico em soros humanos soropositivos para *T. canis* utilizando um Western Blot (WB) com IgG4, foi obtido uma sensibilidade de 70% e especificidade de 100%, evidenciando o potencial de diagnóstico da família de proteína TES em diagnosticar ambas as espécies (Zahabiun et al., 2015).

Contudo, a detecção de IgG4 têm suas desvantagens: enquanto é um anticorpo oriundo de uma defesa mediada por células T auxiliares tipo 2, resposta típica oriunda de uma infecção por *T. canis*, é também o último subtipo de IgG a ser gerado em uma resposta imune, extremamente útil em respostas a infecções crônicas (Aalberse et al., 2009; Holland and Smith, 2006). Sendo assim possível, ter uma baixa

sensibilidade em pacientes em infecções agudas ou até mesmo em crianças, as quais apresentam uma maior incidência e apresentam uma infecção, na maioria das vezes, ainda recentes em comparações a adultos (Araújo et al., 2019).

Embora o ELISA-rTES seja ainda uma incógnita em importantes grupos específicos de populações (crianças, asmáticos e imunodeprimidos, por exemplo), já está sendo desenvolvidos protótipos de testes rápidos para diagnóstico da doença. Tal metodologia permite um diagnóstico imediato e um tratamento rápido com grandes chances de cura (Kundu et al., 2018; Odaga et al., 2014; Wiśniewska-Ligier et al., 2012).

Dentre os métodos imunológicos para diagnóstico rápido, destacam-se o ensaio imunocromatográfico de fluxo lateral (Figura 1) (O'Farrell, 2009). O teste imunocromatográfico de fluxo lateral apresenta operação rápida e simples, com obtenção de resultados imediatos (15 min), baixo custo e o não requerimento de equipamentos sofisticados e caros (Lin et al., 2010; Zhang et al., 2011). Este método tem sido utilizado para o diagnóstico de muitas doenças infecciosas em humanos e animais e para detecção de moléculas bioativas, hormônios, haptenos, entre outros (Heeschen et al., 1998; Lin et al., 2010; Watanabe et al., 2001).

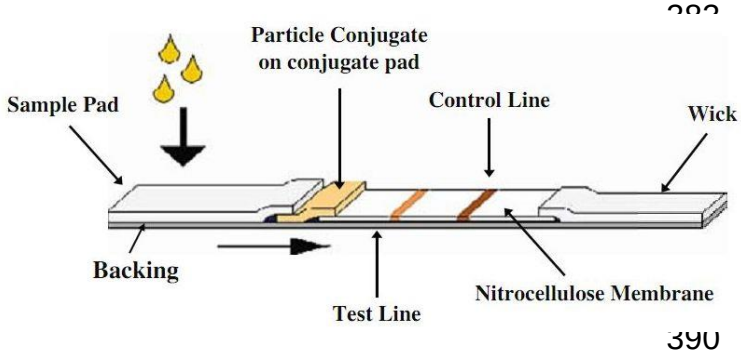

Figura 3. Configuração típica de um ensaio imunocromatográfico de fluxo lateral. Fonte: O'Farrell, 2009.

Em um teste imunocromatográfico lateral, Yunus e colaboradores (2018) utilizaram a rTES-30 e rTES-120, detectadas por IgG4, para diagnosticar toxocaríase em uma população vulnerável. Ao compararem com testes comerciais a base de TES nativo, atingiram sensibilidade de 51.7% a 72.4% (Yunus et al., 2018). Similarmente, em um dispositivo cromatográfico, Lim e colaboradores atingiram 85.7% de

sensibilidade com rTES-30, contudo seu modelo é extremamente sensível a células sanguíneas, requisitando uma filtração do soro prévio ao teste (Lim et al., 2015).

3. HIPÓTESE E OBJETIVOS

3.1 Hipótese

Proteínas recombinantes aplicadas em testes laboratoriais incrementam o atual diagnóstico sorológico de toxocaríase.

3.2 Objetivo Geral

Desenvolver e avaliar um teste imunoenzimático e imunocromatográfico com proteínas recombinantes de *Toxocara canis* para diagnóstico e estudos epidemiológicos da toxocaríase.

3.3 Objetivos Específicos

- Otimizar a expressão dos antígenos TES-30 e TES-120 de *T. canis* em *Escherichia coli* e *Pichia pastoris*;
- Desenvolver um teste imunoenzimático com os antígenos recombinantes;
- Avaliar o teste imunoenzimático;
- Desenvolver um teste imunocromatográfico com os antígenos recombinantes;
- Avaliar o teste imunocromatográfico.

4. CAPÍTULOS

4.1 Artigo 1 – Reactivity of Toxocara canis TES-30 and TES-120 recombinant antigens with sera from mice experimentally infected with *T. canis* and *T. cati*

Artigo publicado na revista *Parasite Immunology*

Title

Reactivity of *Toxocara canis* TES-30 and TES-120 recombinant antigens with sera from mice experimentally infected with *T. canis* and *T. cati*

Running title

Reactivity of rTES-30 & rTES-120 in mice

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Conflict of Interest Statement

This study has no conflict of interest.

Abstract

Aim: While the use of recombinant antigens is being widely investigated in the diagnosis of human toxocariasis, relatively little attention has been given to animal diagnostic models. For this reason, this study aims to investigate the diagnosis potential of *T. canis* TES-30 and TES-120 recombinant antigens in mice, the animal model for toxocariasis studies. **Methods and results**: Serum samples obtained from mice infected with *Toxocara canis* or *Toxocara cati* were tested by indirect ELISA using *T. canis* TES-30 and TES-120 recombinant antigens produced in *Escherichia coli*. 90% of the samples reacted with rTES-30, whereas there was almost no reactivity with rTES-120. **Conclusion**: Despite rTES-120 being a good antigen for diagnosis in humans, it could not reproduce its reactivity in this animal model. Since rTES-30 has good reactivity in mice, it is a valuable tool for diagnosis.

Keywords

Toxocariasis; Diagnosis; Enzyme-Linked Immunosorbent Assay; Blotting, Western; Recombinant Proteins.

Main text

1) Introduction

Toxocariasis is an often-neglected zoonosis with worldwide distribution. The disease is transmitted to animals and humans by infectious eggs from nematodes *Toxocara canis* and *Toxocara cati*. Patients with these infections may develop clinical symptoms that vary with the pattern of larvae migration: coughing, wheezing, and myalgia in visceral larvans migrans; visual impairment and blindness in ocular larvans migrans; encephalitis and meningitis in neurotoxocariasis; and non-specific symptoms in covert toxocariasis[1].

The standard diagnostic method used for this disease is based on laboratory results by Enzyme Linked Immune Sorbent Assay (ELISA), and results are confirmed with Western blotting (in humans) or acid digestion of tissues followed by microscopic visualization (in mice)[2]. Despite both techniques being viable and cost-effective, the production of *T. canis* antigens is arduous, and demands highly specialized laboratory and personnel; requiring a total of 60 days for production. This occurs because it's necessary to cultivate the larvae of the parasite in vitro, restricting even more this disease's diagnostic accessibility[3].

An alternative to native *T. canis* antigen production is to use of recombinant antigens. This shortens the production to 3-4 days and requires a simpler process that is available to most laboratories. Three proteins (rTES-26, rTES-32 and rTES-120) of the secretory and excretory families have been investigated and showed high sensibility and specificity to diagnose toxocariasis in humans[4].

In humans, recombinant proteins have been widely studied for toxocariasis diagnostic, with sensibility and specificity around 70%-100% and 92%-100%, respectively[4–7]. By contrast, there is scarce information in animal studies, especially in

516 mice, where the standard diagnostic methods are still utilized to study various infection
517 paradigms, such as humoral immune response[8–10].

518 For this reason, this study aims to investigate the diagnosis potential of *T. canis*
519 TES-30 and TES-120 recombinant antigens in mice, the animal model for toxocariasis
520 studies.

521

522 **2) Materials and Methods**

523 Synthetic genes of TES-30 (Genbank access 4586556) and TES-120 (Genbank
524 access 1103869) were cloned, using the restriction enzymes *Bam*HI e *Kpn*I, in the
525 expression vector pAE (Invitrogen, USA) with a ligase reaction performed using T4
526 DNA ligase enzyme (Thermo Fisher Scientific, USA). Cloning was confirmed by PCR
527 and sequencing. Recombinant plasmids containing the synthetic genes of TES-30 and
528 TES-120 were used to transform *Escherichia coli* BL21 Star (Invitrogen, USA) that
529 was then cultivated in Luria Bertani (LB) medium (Tryptone 10 g/L, Yeast Extract 5
530 g/L, 170 mM, Synth, Brazil) supplemented with 100 µg/mL of ampicillin (Sigma-Aldrich,
531 USA) at 37 ° C in a rotatory shaker at 180 rpm. When the culture reached an optical
532 density of 0.6 at 600 nm measured by spectrophotometer (Hitachi U-1800, Japan),
533 protein expression was induced for 3 hours at 37 ° C with isopropyl β-D-1-
534 thiogalactopyranoside (Ludwig, Brazil) at the final concentration of 0.5 mM. Chemical
535 lysis with buffer (50 mM Na2HPO4, 500 mM NaCl, pH 8.0, Synth, Brazil) containing
536 10 mg/mL lysozyme (Sigma-Aldrich) and sonication (Cole Parmer, Ultrasonic
537 Homogenizer 4710) were used to release the protein to the medium. After
538 centrifugation (10.000 *g*/10 minutes), the pellet was washed with PBS-T (10 mM
539 sodium phosphate, 150 mM NaCl, 0.05% Tween™ 20, pH 7.5, Synth, Brazil), and
540 solubilized with a 6 M urea phosphate buffer (6 M Urea, 50 mM Na2HPO4, 500 mM

NaCl, 5 mM imidazol, pH 8.0, Synth, Brazil). Proteins were purified with ÄKTA Start and HisTrap HP purification columns (GE Healthcare Life Sciences, USA), using 6 M urea phosphate for buffering and elution (6 M Urea, 50 mM Na2HPO4, 500 mM NaCl, 200 mM imidazol, pH 8.0, Synth, Brazil). The sizes of the expressed target proteins were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were further quantified by Pierce BCA kit (Thermo Fisher Scientific, USA).

Samples were collected from three isolated mice experiments. The animals were donated by the Central Bioterium of the Federal University of Pelotas and Medicine Faculty of the Federal University of Rio Grande. The animals received no treatment and had already been infected prior to the start of this experiment. The animals were orally inoculated through gavage. To recover the adult forms of *T. canis* and *T. cati*, young dogs (pups) and cats were treated with oral Pirantel Pamoate (15 mg/kg). The obtained helminths were identified and sexed; the females were washed in running water and then subjected to uterine tube removal to yield the non-embryonated eggs. These eggs were incubated in 2% formalin at 28 ºC, at greater than 80% humidity, under daily aeration for 30 days until larvae development reached the infecting stage (L3). After incubation, the eggs were refrigerated and then were embryonated specifically to inoculate the animals in this experiment within 1 month of starting refrigeration.

In experiment 1, six 6-week-old Swiss Webster females (*Mus musculus*) were infected with 100 infectious eggs of *T. cati*, and their blood was collected on 0 and 30 days after inoculation (DAI). In experiment 2, seven Swiss 6-week-old Webster females (*Mus musculus*) were infected with 100 infectious eggs of *T. canis*, and their blood was collected on 0 and 30 DAI. In experiment 3, seven 6-week old Swiss Webster

566 females (*Mus musculus*) were infected with 1500 infectious eggs of *T. canis* and their
567 blood was collected on 0, 7, 15, 21 and 28 DAI. A group of two mice was used for
568 control in each experiment. All animals were maintained under controlled temperature
569 with a 12-hour day-night illumination cycle; food, ration and water were *ad libitum*. To
570 confirm infection at day 30, all mice were euthanized so that larvae could be recovered
571 from liver, lungs and brain after digestion with HCl 0.5% (Synth, Brazil) for 24 hours at
572 37 ºC. Sedimental liquid was poured into a centrifuge tube and centrifuged for 10
573 minutes at 1500 rpm, after which 2 ml of the sediment was collected, thoroughly mixed,
574 and parsed into 0.1 ml samples for viewing under the light microscope[11].
575 To verify the antigenicity of the recombinant antigens, we used immunoassay ELISA.
576 The ELISA protocol was optimized prior to the study. Each well of the 96-well flat-
577 bottomed microtiter plate (Nunc Immuno Maxisorp, Thermo Fischer Scientific, USA)
578 was coated with 100 uL of each recombinant antigen at the optimum concentration
579 (50 ng/well) for each antigen in 0.02 M bicarbonate buffer, pH 9.6. The plate was then
580 covered and incubated at 4 °C overnight. The plate was washed three times, for 5
581 minutes each, with PBS-T to remove unabsorbed antigen, after which each well was
582 blocked with 5% dry-milk (Nestle, Sweden) PBS-T solution for 1 hour at 37 °C. The
583 plate was again washed as previously described, and then serum samples were added
584 (100 uL, 1:150 in PBS-T, duplicate wells), and the plate was incubated at 37 °C for 1
585 hour. After the excess serum samples were washed off, monoclonal anti-mouse IgG-
586 horseradish peroxidase (Thermo Fisher Scientific, USA) was added at an optimized
587 dilution (1:2500) in PBS-T and incubated at 37 °C for 1 hour. Following a final washing
588 step, o-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) was added
589 and the ODs were measured after 30 minutes using an ELISA spectrophotometer with
590 absorbance at 450 nm (Biocrom EZ Read 400, United Kingdom). The OD readings

were blanked with the PBS-T, and the cutoff value (based on the results of a ROC statistical analysis) was used to discriminate between the positive and negative results.

Lastly, chemiluminescent Western Blotting (WB) was used to confirm ELISA positivity. The rTES-30 and rTES-120 antigens (5 ug/well) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, USA) via transblot apparatus (Bio-Rad, USA) at 4 °C overnight. The success of the transfer was verified with Ponceau S staining (Sigma-Aldrich, USA). The membrane was cut into strips and blocked with 5% dry-milk PBS-T solution for 1 hour. The strips were then incubated with serum samples (diluted 1:200 in PBS-T) at 4 °C overnight, and then were incubated with either monoclonal anti-human IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) at 1:5000 (in PBST) for 60 minutes or monoclonal anti-mouse IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) at 1:2500 (in PBST) for 60 min. Between each step, the strips were washed with PBS-T for 5 minutes intervals. Finally, the blots were developed with Pierce™ ECL chemiluminescence blotting reagent (Thermo Fischer Scientific, USA) and X-ray films (Thermo Fisher Scientific, USA). Statistical analysis used was two-way ANOVA and ROC curve in GraphPad software.

The studies, using a mouse model, were approved by the ethical committee on animal research, Universidade Federal do Rio Grande (FURG) / Universidade Federal de Pelotas (UFPel) (CEUA protocol no. 23116.004264/2011-00; CEEA protocol no. 7833). The study, using human samples, was approved by the Research Ethics Committee in the Health Area, FURG (protocol no. 102/2012).

3) Results and Discussion

616 This is the first study to show the diagnostic potential of recombinant antigens in
617 mice, the animal model for toxocariasis infection, adopting a simpler protocol using
618 rTES-ELISA in contrast to the more laborious standard method with native TES-ELISA
619 and native TES WB.

620 Overall, rTES-30 had seropositivity in most samples (90%), with the exception of
621 two mice (10%). On the contrary, rTES-120 had close to zero reactivity (Figure 1).
622 While the reactivity of rTES-30 was as expected, since the literature points to rTES-
623 30 being a highly antigenic protein[12], the lack of reactivity of rTES-120 was a surprise.
624 Regarding serum reactivity in our study, we could observe the same immune time-
625 response as when using the native TES protein[13]. All infected mice started to present
626 immune reactivity 15 DAI, achieving peak absorbance at day 28 (Figure 2). Our
627 statistical analysis showed no difference between 14, 21, and 28 DAI ($p>0.05$),
628 confirming that the time gap between days 7 and 14 ($p<0.01$) was the most important
629 regarding humoral response, increasing antibody titers above the established assay
630 cut-off.

631 To confirm the absence of rTES-120's serum reactivity, we used a chemiluminescent
632 Western Blotting assay that can detect up to 0.1 ng of serum antibody concentration
633 (Figure 3)[14]. As a loading/positive control, we used a pool of four human samples that
634 were known to be positive for *Toxocara* spp based on patient history, symptoms,
635 eosinophilia, native TES ELISA (sera were adsorbed with *Ascaris* sp. antigen to avoid
636 cross reactivity), native WB, recombinant ELISA, and recombinant WB[15]. A sera pool
637 (n=4) of non-infected mice from experiments 2 and 3 was used as a negative control.
638 Moreover, in the test lane, we utilized a sera pool (n=4) from infected mice with the
639 largest ELISA absorbances from experiments 2 and 3.

Both protein bands appear clearly in positive lane (human samples), while mice serum only displays the rTES-30 band, with absence of the rTES-120 band. It is important to note that this discrepancy could be caused by the differences between human and mice anti-IgG[16]; however, this is unlikely since rTES-30 had the expected reactivity in both.

Analyzing the sensitivity and specificity in Table 1, we verified that rTES-120 achieved seropositivity in five (25%) of the samples while having overall close to zero absorbance (Figure 1). In addition, no statistical difference (p=0.56) was observed in sensitivity or specificity between the mice infected with lower vs higher doses of infectious eggs. This observation shows the potential of rTES-30 regardless of the infectious dosage.

Different doses in each challenge were used according to Fonseca et al. (2017) and Havasiová-Reiterová et al. (1995)[17,18]. Experiment 1 used a dosage in accordance with experiment 2, validating rTES-30 with *T. cati* infected mice; we decided to not infect mice with a higher dose of *T. cati* infectious eggs because maximum sensitivity had already been achieved with a lower dose. In experiment 2, a low dosage was used to compare antibody response, in *T. canis*, with the high dosage applied in experiment 3. However, no real difference (p=0.49) was seen between the O.D of experiment 2 and 3.

After acid digestion, the mean numbers of larvae found in the brain, lungs, and liver, were 116.7 (sd=21.1), 3 (sd=0.9), and 10.3 (sd=7.8), respectively. These results suggest a major (p=0.03) larvae tropism to the brain. There was no significant difference between different infectious doses of *T. canis* (p=0.31) or protein sensitivity (rTES-30 p=0.26 and rTES-120 p=0.77). These results concur with Fonseca et al.

664 (2017), which did not find a significant influence of parasite inoculum nor humoral
665 immune response[17].

666 In an Iranian study, Nguyen et al. (2017) showed seropositivity of native TES-33,1
667 in mouse and sheep using a Western Blotting assay. While the antigens rTES-26, 32
668 and 33.1 had high homology relation, the reactivity of TES-120 remained unknown[19].
669 Native and recombinant proteins might induce immune response with distinct kinetics,
670 as protein folding is different in each organism[20]. In addition, native TES is highly
671 glycosylated, unlike our proteins, so it should not be expected to have the same
672 reactivity as the native proteins in study, however, deglycosylation of native TES
673 appears to increase sensitivity and specificity as reported by Roldán et al. (2015)[21,22].
674 This information helps explain the good performance of the antigens produced in *E.*
675 *coli*, an expression platform incapable of performing protein glycosylation.

676 Most studies that involve the use the recombinant antigens in toxocariasis diagnosis
677 use both rTES-30 and rTES-120 as predicted models with high antigenic potential.
678 Albeit human samples have shown a potential response for rTES-30 and rTES-120,
679 as published by Mohamad et al (2009), Zahabiun et al (2015), Fong et al (2003) and
680 Peixoto et al. (2011), in mouse, the animal model for this disease, rTES-120 didn't
681 prove itself as an antigenic protein (Figure 1)[4–7]. Sperotto et al (2017) published a
682 proteomic analysis of *in vitro T. canis* larvae and identified both proteins, rTES-30 and
683 rTES-120, indicating that larvae cultivated *in vitro* express them both[23]. As the
684 antibodies against TES-120 protein is produced in humans (Figure 3), the lack of
685 reactivity in mice suggest that this antigen is not as immunogenic in mice, or the larvae
686 doesn't express the protein in the animal, changing its pathogeny from species to
687 species. Remains unknown if any other animal species present this protein-antibody
688 divergence, or if this is a mice isolated case.

In conclusion, while we did not achieve 100% sensitivity with *T. canis*, we present rTES-30 as a viable alternative to native TES with recombinant ELISA; we cannot recommend rTES-120 as it did not prove itself to be an adequate diagnostic tool. The rTES-30 alternative has multiple advantages over antigen production and fewer cross-reactivity issues. Therefore, we strongly suggest the use of rTES-30 for animal model studies.

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784 **Tables**

785

786 Table 1. Diagnosis potential of rTES-30 and rTES-120 in mice infected by *Toxocara*
787 *canis* (n=14) and *Toxocara cati* (n=6)

Experiment	**Parameter**	**rTES-30**	**rTES-120**
1	Sensitivity	100%	16.7%
Toxocara cati	Specificity	100%	100%
100 eggs	Cut-off†	0.10	0,08
2	Sensitivity	85.7%	28.6%
Toxocara canis	Specificity	100%	85.7%
100 eggs	Cut-off†	0.06	0,05
3	Sensitivity	85.7%	28.6%
Toxocara canis	Specificity	100%	100%
1500 eggs	Cut-off†	0.07	0,01

788 † Cut-off chosen by ROC Curve analysis

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Figure Legends

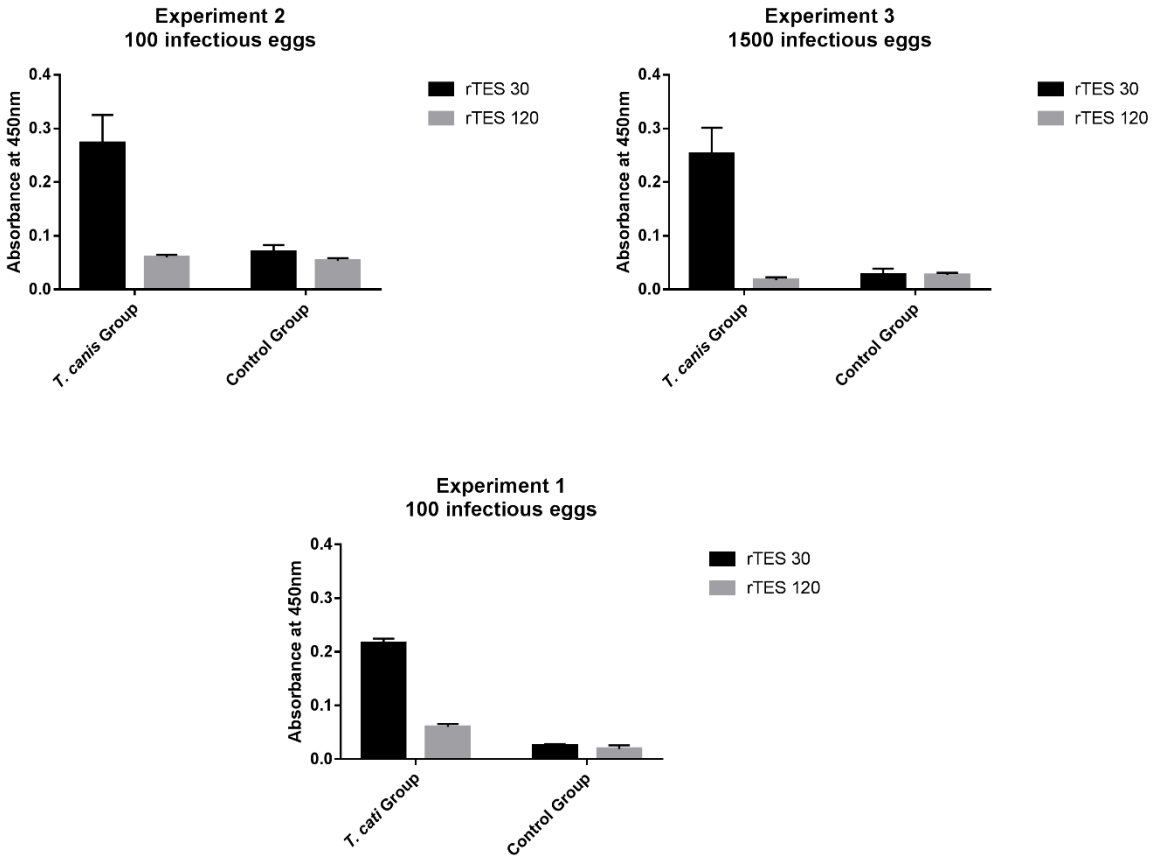

Figure 1. ELISA absorbance comparison between mice infected by *Toxocara canis* (Experiment 2 and 3) (n=14) and mice infected by *Toxocara cati* (Experiment 1) (n=6) at ~30 days after infection using recombinant proteins rTES-30 and rTES-120.

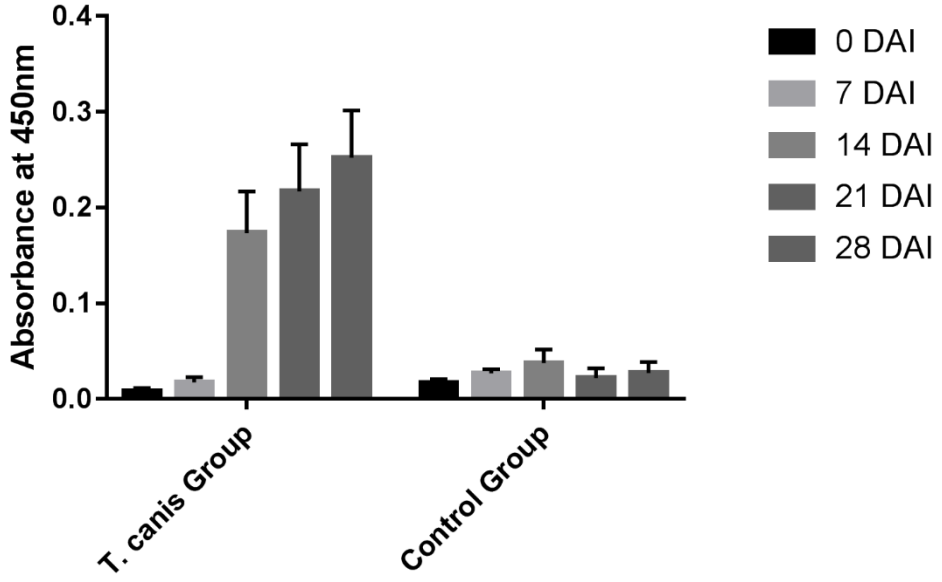

Figure 2. ELISA absorbance analysis between day after infection (DAI) in mice inoculated with 1500 infectious eggs of *Toxocara canis* (n=7) using rTES-30.

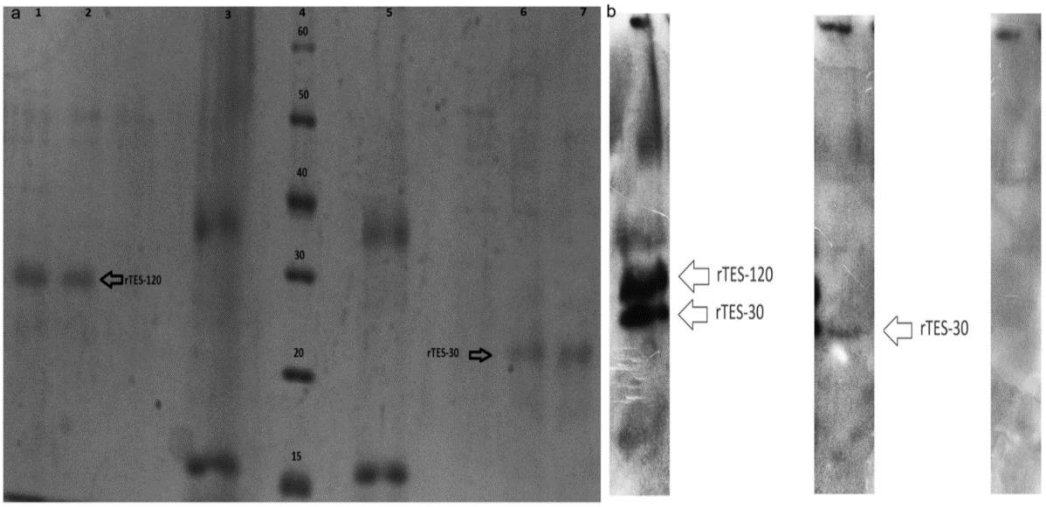

Figure 3. a) SDS-PAGE post AKTA purification; lane 1: rTES-120 elution fraction 1; lane 2: rTES-120 elution fraction 2; lane 3: Flow through of rTES-120; lane 4: BenchMark™ His-tagged Protein Standard (Thermo Fischer Scientific, (USA); lane 5: Flow through of rTES-30; lane 6: rTES-30 elution fraction 1; lane 7: rTES-30 elution fraction 2. b) Chemiluminescent Western Blotting: positive control lane (left); mice sera test lane (middle); and negative control lane (right) with rTES-30 and rTES-120.

4.2 Artigo 2 – Sensitivity and specificity of recombinant proteins in Toxocara spp. for serodiagnosis in humans: differences in adult and child populations.

Artigo publicado na revista *Plos One*

Sensitivity and specificity of recombinant proteins in *Toxocara* spp. for serodiagnosis in humans: differences in adult and child populations.

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Abstract

Toxocariasis is a neglected zoonosis that affects children and adults. Recombinant proteins have been widely investigated for diagnosis, achieving high sensitivity and specificity in an overall population; however, little is known about age as a factor in its application. This study aims to investigate the diagnostic potential of *Toxocara canis* TES-30 and TES-120 recombinant proteins in humans, differentiating between its performance in children and adults. Serum samples collected from children and adults seropositive to *Toxocara* spp. were tested with indirect ELISA using *T. canis* TES-30 and TES-120 recombinant proteins produced in *Escherichia coli*. While rTES-30 sensitivity was not affected by age (81.8% in children and 87% in adults), rTES-120 sensitivity severely decreased in children to only 63.6%, down from 95.7% in adults. Furthermore, the sensitivity of rTES-30 increased to 97.8% after Western blotting confirmation. High specificity (>94%) against other geohelminths was reported for both recombinant proteins. Our study favors the use of rTES-30 with total IgG as the primary antibody in an indirect ELISA assay as a tool for epidemiological human studies.

Introduction

According to researchers at the Centers for Disease Control, toxocariasis is one of the most common neglected diseases in populations living in poverty (1). This disease is transmitted via the infectious eggs of *Toxocara* sp. to definitive (dogs and cats) and paratenic (humans) hosts. In definitive hosts, the larvae mature to parasite adults and complete its life cycle: the host excrete non-infective eggs on the soil, which become infectious in the environment and is made available for infect new definite or paratenic hosts; however, in paratenic hosts, the larvae cannot reach adulthood, therefore, it

migrates to tissue and organs and remain in the host until the immune system clear the infection or forces them into a state of arrested development (1). For this reason, its symptomatology, in humans, varies according to larvae migration and its damage to the tissues; visceral larva migrans syndrome and covert toxocariasis are more common in children, while ocular toxocariasis and neurotoxocariasis can be found in both children and adults (2–5).

Clinical diagnosis is difficult as most manifestations (excluding ocular toxocariasis) lack characteristic symptoms, so the only viable diagnostic option is in the laboratory with Enzyme-linked immunosorbent assay (ELISA) and Western Blotting (6). The production of proteins to be used in the laboratory assays requires in vitro larvae cultures, which demand excessive time, cost, and expertise to produce a viable product. Also, the *Toxocara* excretion-secretion protein family (TES) obtained from the larvae culture presents a number of cross-species reactions, requiring serum preabsorption that is troublesome in itself (7).

As an alternative, multiple studies have investigated the potential of recombinant proteins in the diagnosis of toxocariasis to reduce the time, cost, and cross-reactivity of native TES production (8–11). Of the recombinant proteins previously studied, TES-30 and TES120 have been the most promising, achieving high sensitivity and specificity in the general population (8).

In children, however, these recombinant proteins have not been investigated thoroughly. Children are substantially more exposed to one of the most important risk factor for the disease: the geophagy habit. In addition, children are at risk for early infection and progression to ocular toxocariasis or/and neurotoxocariasis, potentially conferring permanent damage to vision and/or the brain, respectively, implicating toxocariasis as a major non-diagnosed health problem (4,12,13). In addition, prior

research has noted that the immune system of children responds differently than the immune system of adults to parasite infection, with lower expression levels of toll-like receptors and interleucin-5, both of which are important signaling pathways in the mature response against parasites (14–16). This discrepancy in immune response could cause a deviation from the high sensitivity and specificity observed in adult studies.

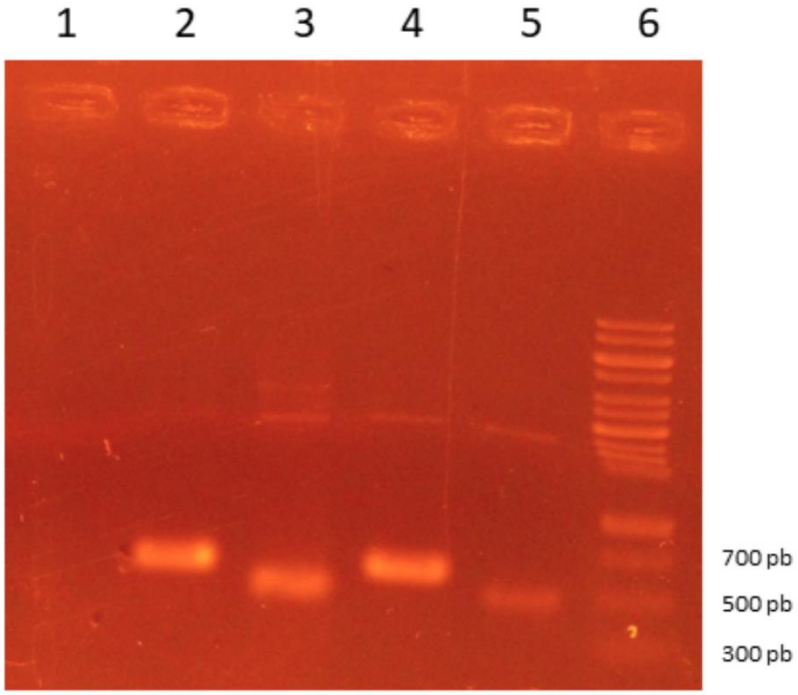
Therefore, this study investigates the diagnosis potential of *T. canis* TES-30 and TES-120 recombinant proteins in children versus adults with the objective of validating an inexpensive diagnosis in the pediatric population. If effective, this diagnostic technique would represent an essential step for combating neglected aspects of the disease while permitting a fast and accurate diagnosis to reduce or prevent poor prognoses.

Materials and Methods

Cloning and Expression of Recombinant Proteins

Synthetic genes of TES-30 (Genbank access 4586556) and TES-120 (Genbank access 1103869) were cloned, using the restriction enzymes *Bam*HI e *Kpn*I, in the expression vector pAE (17) with a ligase reaction performed using T4 DNA ligase enzyme (Thermo Fisher Scientific, USA). Cloning was confirmed by PCR (Fig 1) and sequencing, primer details are available in table 1. Recombinant plasmids containing the synthetic genes of TES-30 and TES-120 were used to transform *Escherichia coli* BL21 Star (Invitrogen, USA) that was then cultivated in Luria Bertani (LB) medium (Tryptone 10 g/L, Yeast Extract 5 g/L, 170 mM, Synth, Brazil) supplemented with 100 µg/mL of ampicillin (Sigma-Aldrich, USA) at 37 ° C in a rotatory shaker at 180 rpm.

When the culture reached an optical density of 0.6 at 600 nm measured by spectrophotometer (Hitachi U-1800, Japan), protein expression was induced for 3 hours at 37 ° C with isopropyl β-D-1-thiogalactopyranoside (Ludwig, Brazil) at the final concentration of 0.5 mM. Chemical lysis with buffer (50 mM Na2HPO4, 0.5 M NaCl, pH 8.0, Synth, Brazil) containing lysozyme (Sigma-Aldrich) and sonication (Cole Parmer, Ultrasonic Homogenizer 4710) were used to release the protein to the medium. After centrifugation (10.000 *g*/10minutes), the pellet was washed with PBS-T (10 mM sodium phosphate, 0.15 M NaCl, 0.05% Tween™ 20, pH 7.5, Synth, Brazil), and solubilized with a 6 M urea phosphate buffer (6 M Urea, 50 mM Na2HPO4, 0.5 M NaCl, 5 mM imidazole, pH 8.0, Synth, Brazil). Proteins were purified with ÄKTA Start and HisTrap HP purification columns (GE Healthcare Life Sciences, USA), using 6 M urea phosphate buffer and 6 M urea elution phosphate buffer (6 M Urea, 50 mM Na2HPO4, 0.5 M NaCl, 200 mM imidazol, pH 8.0, Synth, Brazil). The sizes of the expressed target proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The proteins were further quantified by Pierce BCA kit (Thermo Fisher Scientific, USA).



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952 **Fig 1. Agarose gel stained with ethidium bromide presenting PCR products.**
953 Lane 1: PCR Negative Control (PCR Mix). Lane 2: pAE rTES-30 Clone 1. Lane 3: pAE
954 rTES-120 Clone 1. Lane 4: pAE rTES-30 Clone 2. Lanes 5: pAE rTES-120 Clone 2.
955 Lanes 6: Ladder 100 pb (Ludwig Biotecnologia, Brazil).

956

957 Table 1. Primer details of rTES-30 and rTES-120 for confirmation of cloning in
958 expression vector pAE.

Protein	Forward Primer Sequence	Reverse Primer Sequence	Size of Product (pairs of base)
rTES-30	5'-CCCAAGCTTTCACAAAGGTCTCTTACA–3'	5'-CCCAAGCTTTCACAAAGGTCTCTTACA–3'	641
rTES-120	5'-AGCGGATCCATGTCTTCTTCTTCTTC–3'	5'-TTCAAGCTTCTAGCAGAATCCACAGGTCAA–3'	502

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Sample Collection

Samples were collected from three distinct serum panels. Serum panel 1 samples were collected from 48 children between 2-8 years old at the Hospital Universitário by Dr. Miguel Riet Corrêa Jr in Rio Grande - RS, Brazil. Within this collection, 22 children were seropositive for *Toxocara* spp. infection and 26 children were seronegative. Positives and negatives samples were diagnosed by clinic data, seropositivity to *Toxocara* spp. (via native TES ELISA and Western blotting confirmation) and three epidemiological factors: eosinophilia, contact with young dogs/cats, and geophagy. Serum panel 2 were collected samples from 42 adults (age > 21 years) at the Parasitology Laboratory of the Universidade Federal do Rio Grande and Universidade Federal de Pelotas. 23 adults were seropositive for *Toxocara* spp. infection and 19 seronegatives were adults. Positives and negatives samples were diagnosed by clinic data, seropositivity to *Toxocara* spp. (via native TES ELISA and Western blotting confirmation) and two epidemiological factors: eosinophilia and contact with young dogs/cats. Serum panel 3 samples were collected from Laboratório de Soroepidemiologia e Imunobiologia do Instituto de Medicina Tropical de São Paulo (IMT-SP) of Universidade de São Paulo. All sera were found to be seronegative for *Toxocara* spp. infection based on epidemiology factors and laboratory assays. Other parasites were diagnosed from clinic data, epidemiology, and laboratory assays, revealing 40 cases with parasite infections (9 patients with *Ascaris lumbricoides ,* 2 patients with *Trichuris trichiura,* 4 patients with *Ancylostoma duodenale,* 16 patients with *Strongyloides stercoralis,* 6 patients with *Hymenolepis nana,* and 3 patients with *Fasciola hepatica*) and 40 cases without.

Indirect ELISA

To verify the antigenicity of the recombinant proteins, we used ELISA. ELISA protocol was optimized prior to the study. Each well of the 96-well flat-bottomed microtiter plate (Nunc Immuno Maxisorp, Thermo Fischer Scientific, USA) was coated with 100 µL of each recombinant antigen at the optimum concentration (50 ng) for each antigen in 0.02 M bicarbonate buffer, pH 9.6. The plates were then covered and incubated at 4°C overnight. The plates were washed with PBS-T, to remove unadsorbed antigen. After a washing step of three washes for 5 min each with PBS-T, each well was blocked with 5% dry-milk (Nestle, Sweden) PBS-T solution for 1 h at 37°C. The plates were again washed as previously described, followed by the addition of sera samples (100 µL, 1:150 in PBS-T, duplicate wells), and incubated at 37°C for 1 h. After the washing step, monoclonal anti-human IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) were added at an optimized dilution (1:5000) in PBS-T, or mouse anti-human IgG2 (Sigma Aldrich, USA) were added at an optimized dilution (1:2500) in PBS-T, or mouse anti-human IgG4 (Sigma Aldrich, USA)) were added at an optimized dilution (1:2500) in PBS-T, followed (in case of IgG2 and IgG4) by anti-mouse IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) and incubated at 37°C for 1 hour. Following a final washing step, o-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) was added and the ODs were measured after 15 min as absorbance at 450 nm using an ELISA spectrophotometer (Biocrom EZ Read 400, United Kingdom). The OD readings were blanked with the PBS-T, and the cutoff value was used to discriminate between the positive and negative results. This cutoff value was based on the results of a ROC statistical analysis: 0.3415 for rTES-30/IgG; 0.23 for rTES-120/IgG; 0.083 for rTES-30/IgG2; 0.093 for rTES-120/IgG2; 0.076 for rTES-30/IgG4; and 0.0965 for rTES-120/IgG4.

Western Blotting Assay

Lastly, a Western Blotting assay was used to confirm ELISA close to cut-off absorbance (borderline). The rTES-30 (20 µg/ml) was applied in a 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, USA) by using a transblot apparatus (Bio-Rad, USA) at 4°C overnight. Protein transfer to membrane was checked with Ponceau S staining (Sigma-Aldrich, USA). The membrane was cut into strips and blocked with 5% dry-milk (Nestle, Sweden) PBS-T solution for 1 h. The strips were then incubated with serum/sera samples (diluted 1:200 in PBS-T) at 4°C overnight, followed by monoclonal anti-human IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) were added at an optimized dilution (1:5000) in PBS-T and incubated at 37°C for 1 hour. Between each step, the strips were washed with PBS-T during 5 minutes. Finally, DAB Solution (0.025% 3,3′-Diaminobenzidine, 0.0009% H_2O_2 and 0.05M Tris/HCl-solution, Sigma Aldrich, USA) were used to develop the blots. Statistical analysis was performed by Pearson chi-square, two-way ANOVA and ROC curve in GraphPad software.

Ethics

This study was approved by the Research Ethics Committee in the Health Area, FURG (protocol no. 102/2012 and 100/2010).

Results and discussion

The expression of our recombinant proteins yielded an 18 kDa protein (rTES-30) and a 24 kDa protein (rTES-120) obtained in inclusion bodies at the concentrations

of 17.04 mg/L and 28.56 mg/L, respectively. This protein was stored in a ready-to-use state in urea buffer, similar to Farmer et al. (2017) study (19).

The protein bands were confirmed in a Western Blotting assay. We observed a higher purity of recombinant protein than was published by Mohamad et al. (2009) and Anderson at al. (2015). We achieved this purity by inserting a double histidine tag at each extremity of the protein (5' and 3'), increasing its affinity to the chromatography column (8,20).

The overall sensitivity and specificity of recombinant proteins is shown in Table 2 and Table 3. There was no benefit associated with using both proteins at the same time, as only one sample reacted with rTES-120 and not with rTES-30 (p=0.8152).

Table 2. Optimum sensibility and specificity for each protein and antibody after Receiver Operating Characteristic (ROC) analysis.

Protein/Antibody	Positive Samples (Sensitivity)	Negative Samples (Specificity)
rTES-30/IgG	38/45 (84.4%)	45/45 (100%)
rTES-30/IgG2	4/45 (8.9%)	40/45 (88.9%)
rTES-30/IgG4	10/45 (22.2%)	44/45 (97.8%)
rTES-120/IgG	34/45 (75.6%)	45/45 (100%)
rTES-120/IgG2	6/45 (13.3%)	43/45 (95.6%)
rTES-120/IgG4	23/45 (51.1%)	44/45 (97.8%)

Table 3. Specificity of recombinant proteins against a variety of common geohelminths.

	Cross-Reactivity[a]					
	Ascaris lumbricoides	*Trichuris trichiura*	Ancylostomids	*Strongyloides stercoralis*	*Hymenolopis nana*	*Fasciola hepatica*
rTES-30	0/9 0%	0/2 0%	0/4 0%	1/16 6.25%	1/6 16.67%	0/3 0%
rTES-120	2/9 22.22%	0/2 0%	0/4 0%	0/16 0%	1/6 16.67%	0/3 0%

a) Cut-off for each antigen was calculated by ROC curve assay of 40 *Toxocara* spp. negative patients.

After categorizing the samples by age, we observed a major change in sensitivity in some protein/antibody combinations, as shown in Table 4.

Table 4. Sensitivity and specificity for each protein and antibody after ROC analysis, separated by adult and child samples.

Protein/Antibody	Adult Positive Samples (Sensitivity)	Adult Negative Samples (Specificity)	Children Positive Samples (Sensitivity)	Children Negative Samples (Specificity)
rTES-30/IgG	20/23 (87%)	19/19 (100%)	18/22 (81.8%)	26/26 (100%)
rTES-30/IgG2	1/23 (4.3%)	18/19 (94.7%)	1/22 (4.5%)	25/26 (96.2%)
rTES-30/IgG4	6/23 (26.1%)	19/19 (100%)	1/22 (4.5%)	26/26 (100%)
rTES-120/IgG	22/23 (95.7%)	19/19 (100%)	14/22 (63.6%)	26/26 (100%)
rTES-120/IgG2	2/23 (8.7%)	18/19 (94.7%)	4/22 (18.2%)	25/26 (96.2%)
rTES-120/IgG4	16/23 (69.6%)	19/19 (100%)	6/22 (27.3%)	26/26 (100%)

In this study, we analyzed the diagnostic potential of *T. canis* recombinant proteins in more detail, addressing questions raised by previously studies regarding

the use of antibody classes for TES antigens and the effects of patient age on sensitivity and specificity.

Overall, we disagree with Mohamad et al. (2009) and Watthanakulpanich et al. (2008), both of which find a higher sensitivity using a combination of proteins to serodiagnose *Toxocara* spp. infections (8,21), while our study favors the use of only one protein (rTES-30). We found that sensitivity and specificity did not improve with the combination of both proteins (p=0.8152), so we recommend the single protein to reduce the associated production cost.

Another difference is that we found a lower sensitivity associated with IgG4 and IgG2, while previous studies found these to be the most sensitive and specific antibodies for toxocariasis. This discrepancy could be attributable to differences in population; this study was performed in Brazil with a climate, culture, and genetic features of *Toxocara* spp. that likely are different in Malaysia, Thailand, and Scotland (8,21,22). Moreover, we opted to use total IgG instead of subgroups to reduce the cost of diagnosis, as total IgG is less expensive than a specific IgG subgroup (from any given manufacturer).

We also observed a major increase in IgG4 sensitivity in adults over children for both rTES-30 (4.5% in children to 26.1% in adults, p=0.0959) and rTES-120 (27.3% in children to 69.6% in adults, p=0.0072). This variable could explain why Mohamad et al. (2009) found that IgG4 was the most sensitive: that study used an adult population. Among all immunoglobulins, IgG4 is produced the least and is the last to be produced, so it is positive in chronic diseases only (23). As children do not yet have a mature immune system and are more likely to have contracted the infection recently, the immune system has less time to produce a viable IgG4 mediated-response (14,23).

In addition, Watthanakulpanich et al. (2008) used native TES to infer that IgG2 was the most sensitive and specific. Native TES is highly glycosylated, unlike in our expression system (*Escherichia coli*), so it cannot be expected to induce the same reactivity. Also, Watthanakulpanich et al. (2008) did not preabsorb serum with *Ascaris* sp. so cross-reactivity with other geohelminths could have influenced the results (21,24).

Regardless, our main finding is the difference in sensitivity between adult and pediatric age groups. When we divided our sample pool between children (age 2-8 years) and adults (age > 21 years), we discovered that rTES-120 sensitivity correlated negatively with age, decreasing from 95.7% in adults to 63.6% in children (p=0.0098). Meanwhile, rTES-30 sensitivity was not affected by age: 81.8% in children and 87% in adults (p= 0.6995).

Native TES-120 is a mucin, a heavily glycosylated protein, so its lower sensitivity in children could mimic the situation that occurs in encapsulated bacteria vaccinations, such as those for *Haemophilus influenzae*, *Streptococcus pneumoniae,* and *Neisseria meningitidis*. The polysaccharides of these bacteria in isolation do not illicit a proper immune response in young children because they lack a mature T-independent response. Mucin has a similarly high concentration of carbohydrates as polysaccharides, so this could explain the discrepancy we found in specificity between children and adults (25–27). Alternatively, native TES-30 is a C-type lectin, with serine proteinase activity and a lower ratio of carbohydrates to protein than native TE-120. It is associated with the larval migratory process and infection; hence, it is one of the first proteins to be produced and recognized by the pediatric immune system, explaining its higher sensitivity (24,28).

In this study, we defend the use of rTES-30 as a sole recombinant protein for the serodiagnosis of human *Toxocara* spp. We achieved an 84.4% (38/45) sensitivity with ELISA regardless of the patient's age and reached 97.8% (44/45) with Western Blotting confirmation (Fig 2). The discrepancy between ELISA and Western Blot results was expected as ELISA determines that a sample is positive or negative based on an arbitrary number (cut-off), while a Western Blotting assay relies on a simple observation of an adequate size band (29,30). We suggest that ELISA is still the optimal first-line technique because it is relatively inexpensive, making it a good fit for a neglected disease (31), but a Western Blotting assay should be utilized as a confirmation technique in sera with absorbances that are close to the cut-off, this has also been suggested by Maraghi et al. (2012) (32). We also recommend a Western Blotting assay to rule out other causes (infectious or non-infectious) for a specific symptom or finding, such as hypodense or hyperintense brain lesions (neurotoxocariasis), and to evaluate immunocompromised patients (e.g., to rule out toxoplasmosis, schistosomiasis, and others common opportunistic diseases) (2,33–36).

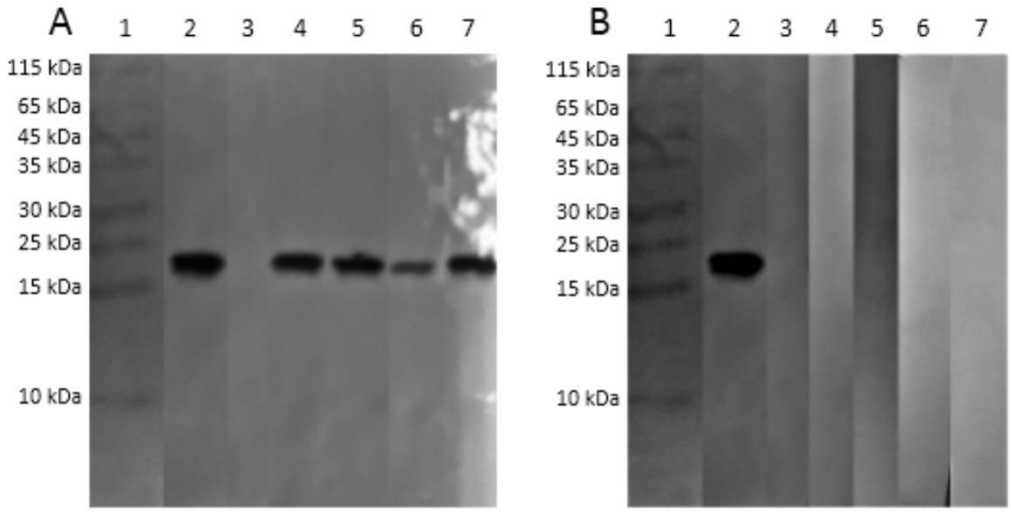

Fig 2. (A) Western blot analysis of rTES-30 antigen probed with serum that was close to the ELISA cut-off. Lane 1: PageRuler™ Prestained Protein Ladder (Thermo Fischer Scientific, USA). Lane 2: positive/loading control. Lane 3: negative control. Lanes 4-7: sera from four different Toxocara-seropositive patients. **(B) Western blot analysis of rTES-30 antigen probed with serum that was close to the ELISA cut-off.** Lane 1: PageRuler™ Prestained Protein Ladder (Thermo Fischer Scientific, USA). Lane 2: positive/loading control. Lane 3: negative control. Lanes 4-7: sera from apparently healthy people.

Moreover, even with an ELISA sensitivity of 95.7% in adults, no statistical difference was seen between rTES-120 and rTES-30 (p=0.6078). Our finding is confirmed by a recent study from the Centers for Disease Control and Prevention (CDC). A U.S. national survey performed with recombinant rTc-CTL-1 (99.9% identity to rTES-30) found a 5.1% prevalence in one developed country (19). That study used a Luminex bead assay, similar to but more expensive than an indirect ELISA as the protein rTc-CTL-1 needed to be pre-coated with color-coded beads.

Furthermore, we expected high specificity (>94%) of every recombinant protein as has been documented previously (8). It is important to state that we expect that the recombinant proteins have cross-reactivity with *T. cati* TES (9), homolog to *T. canis*, this cross-reactivity is not a specificity issue and it is important that both recombinant proteins detect *T. cati* sorology, as the infection remains the same with potential to cause visceral and ocular toxocariasis (37). While Mohamad et al. (2009) studied the specificity of recombinant proteins against *Ascaris lumbricoides*, *Trichuris trichiura*, lymphatic filariasis, amoebiasis, and toxoplasmosis, we studied the specificity of recombinant proteins against strongyloidiasis, hymenolepiasis, and fasciolosis—three diseases that affect patients of developing countries and could potentially jeopardize the diagnosis of toxocariasis with native TES (8,38,39). Strongyloidiasis is particularly noteworthy as *Strongyloides stercoralis* and *Toxocara* sp. are endemic parasites in tropical countries (e.g., Brazil) and are often co-morbid in patients with human immunodeficiency virus and neuroparasites. In these situations, it is therefore ideal to have a diagnostic assay without cross-reactivity issues (33,40–42).

Conclusions

In conclusion, our study favors the use of rTES-30 with total IgG as the primary antibody in an indirect ELISA assay as a tool for epidemiological human studies.

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4.3 Manuscrito 1 – Evaluation of *Toxocara canis* glycosylated TES produced in *Pichia pastoris* for immunodiagnosis of human toxocariasis

Manuscrito submetido à revista *Brazilian Archives of Biology and Technology*

i. Title

Evaluation of *Toxocara canis* glycosylated TES produced in *Pichia pastoris* for immunodiagnosis of human toxocariasis

ii. Running Title

Toxocariasis diagnosis with *Pichia* TES

iii. Abstract

Toxocariasis is a zoonotic disease that can infect humans and induce irreversible lesions. Recombinant proteins are a suggested alternative for the diagnosis of Toxocara canis infection. The current Escherichia coli recombinant protein overexpression system usually produces insoluble products. As an alternative, yeast such as Pichia pastoris have secretory mechanisms, which could diminish the cost and time for production. This study aimed to produce recombinant proteins in Pichia pastoris and verify their sensibility and specificity in an indirect ELISA assay. Two sequences (rTES-30 and rTES-120) of Toxocara canis excretory-secretory antigens were cloned in a pPICZαB vector and expressed in P. pastoris KM71H. Sera samples collected from human adults infected by Toxocara spp. were tested by indirect ELISA using rTES-30 and rTES-120 as antigens. Recombinant proteins were detected at 72 hours after induction, in the supernatant, as pure bands between 60~70 kDa with hyperglycosylation. Regarding diagnosis potential, recombinant antigens had high specificity (95.6%); however, sensitivity was 55.6% for rTES-30 and 68.9% for rTES-120. Further deglycosylation of the P. pastoris antigens did not seem to affect ELISA performance (p>0.05). The sensitivity in the serodiagnosis diminished any advantage that P. pastoris expression could have. Therefore, we do not recommend P. pastoris recombinant TES production as an alternative for the diagnosis of toxocariasis.

iv. Key-words

Diagnosis; ELISA; Recombinant Protein; Glycosilation.

1. INTRODUCTION

Toxocariasis is a neglected zoonosis with a worldwide distribution and is transmitted to animals and humans by infectious eggs from *Toxocara canis* and *Toxocara cati*. Infected patients may develop clinical symptoms according to larvae migration, obscuring or even invaliding clinical diagnosis. The traditional diagnostic method for this disease relies on Enzyme Linked Immune Sorbent Assay (ELISA) and Western Blotting[1]. While these laboratory methods are viable and cost-effective, the production

of *T. canis* antigens is a major disadvantage that restricts its accessibility because the antigen production is arduous and requires a minimum of 60 days[2].

As an alternative, recombinant proteins of the secretory and excretory families (TES) have been proposed. Three recombinant proteins (rTES-26, rTES-32, and rTES-120) were produced in *Escherichia coli* and showed high sensibility and specificity, essential requirements for the diagnosis of toxocariasis in humans[3]. However, *E. coli* express TES proteins in the cytoplasm and may therefore generate insoluble products, impeding purification treatments and increasing the associated production cost[4].

Expression in *Pichia pastoris* is an interesting alternative to recombinant TES produced in *E. coli*. One advantage is the secretory mechanism presented by yeast, which serves as an *in vivo* purification methodology and reduces the major costs associated with purifying recombinant proteins. Also, *E. coli* produce TES recombinant proteins in inclusions bodies, which require denaturing conditions and posterior refolding that is evitable in *P. pastoris*[3,5]. Another advantage is that *P. pastoris* can be cultivated in a low-cost medium at a broader pH range and lower temperatures—essential characteristics for industrial scale[6]. Finally, the last advantage over the *E. coli* system is the possibility of post-translational modifications, especially in the case of native TES, as the proteins are highly glycosylated and could respond to a part of the immune response[7]. Prior to this study, Fong et al. presented the recombinant TES-120 expressed in *P. pastoris* with high specificity, however they did not studied the sensitivity, suggesting an unexplored potential for TES protein in *Toxocara* spp. infection diagnosis[8].

For this reason, this study aims to produce recombinant proteins (rTES-30 and rTES-120) in the yeast *Pichia pastoris* and verify their sensibility and specificity in an indirect ELISA assay.

2. MATERIALS AND METHODS

2.1. Cloning and Expression of Recombinant Proteins

To induce the expression of the TES-30 and TES-120 antigens, synthetic genes (Epoch Biolabs, Inc. USA) containing *Pichia pastoris* preferential codons and restriction enzyme sites were synthesized to direct cloning into the expression vector *P. pastoris* pPICZαB (Invitrogen. USA) according to the sequences deposited in GenBank under accession numbers 4586556 (TES-30) and 1103869 (TES-120).

Synthetic genes were cloned into the pPICZαB vector according to Green & Sambrook[9]. A recombinant clone from each construct was linearized with restriction enzyme *Pme*I to optimize vector integration by homologous recombination. *Pichia pastoris* KM71H (MutS) was grown on an orbital shaker at 28 °C and 120 rpm. Competent cells were transformed by electroporation with 10 µg of the linearized recombinant vector (pPICZαB-TES-30 and pPICZαB-TES-120) according to Invitrogen (EasySelectTM Pichia Expression Kit, Version G; Invitrogen, USA). The recombinant clones were selected on YPD agar (bacteriological peptone 20 g/L, yeast extract 10 g/L, glucose 20 g/L, 15 g/L Agar; Synth, Brazil) with Zeocin 500 ug/mL after incubation for four days at 28 °C.

Transformants were spiked in BMGY broth (2.0% peptone, 1.0% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.4 µg/mL biotin, 1.0% glycerol; Synth, Brazil) and incubated in the orbital agitator (100 rpm at 28 °C) until they reached DO_{600}=4. At that time, the culture was centrifuged and the pellet was resuspended in BMMY (2% peptone, 1% yeast extract, 1.34% potassium phosphate, pH 6.0, 100mM yeast nitrogen base without amino acids, 0.4

μg/mL biotin, 0.5% methanol; Synth, Brazil) containing 0.5% methanol. For 7 days, 0.5% methanol was added every 24 hours to the culture of the clones containing pPICZαB-TES-30 and pPICZαB-TES-120. Samples of supernatant and pellet were collected daily. A well-characterized *P. pastoris* clone (pPICZαB-gD) was cultivated as a positive control[10] and native *P. pastoris* Km71H was cultivated for a negative control; both performed under the same culture conditions described previously. The rTES-30 and rTES-120 proteins were concentrated from the culture supernatant by dialysis (Membra-cell MD34, Viskase, USA) in high osmolarity solution (5M dextrose, Synth, Brazil).

2.2. Deglycosylation

A fraction of protein volume was submitted to a deglycosylation procedure following the protocol described by Promega (Endoglycosidase H. Promega, USA). Expression levels and digestion were analyzed in the supernatant by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The proteins were further quantified with the Pierce BCA kit (Thermo Fisher Scientific, USA).

2.3. Sample Collection

Samples were collected from two distinct blood banks. Blood bank 1 donated samples that were collected from 90 health workers at the Hospital Universitário Dr. Miguel Riet Corrêa Jr in Rio Grande - RS, Brazil and at the Parasitology Laboratory of the Universidade Federal de Pelotas between the years of 2014-2015. Within this collection, 45 health workers were positive for *Toxocara* spp. infection and 45 were negative. Infection was diagnosed by seropositivity to *Toxocara* spp. (via native TES ELISA and Western blotting confirmation) and three epidemiological factors: eosinophilia, contact with young dogs/cats, or geophagy. Blood bank 2 donated 80 negative to *T. canis* samples from the Laboratório de Soroepidemiologia e Imunobiologia do Instituto de Medicina Tropical de São Paulo (IMT-SP) of Universidade de São Paulo. All sera were found to be negative for *Toxocara* spp. infection based on epidemiology factors and laboratory assays. Other parasites were diagnosed from clinic data, epidemiology, and laboratory assays, revealing 40 cases with parasite infections (*Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostomids*, *Strongyloides stercoralis*, *Hymenolepis nana*, and *Fasciola hepatica*) and 40 cases without. All sera were stored at -20C until laboratory assay.

2.4. Indirect ELISA

To verify the antigenicity of the recombinant proteins, we used ELISA. ELISA protocol was optimized prior to the study. Each well of the 96-well flat-bottomed microtiter plate (Nunc Immuno Maxisorp, Thermo Fischer Scientific, USA) was coated with 100 uL of each recombinant antigen at the optimum concentration (50 ng) for each antigen in 0.02 M bicarbonate buffer, pH 9.6. The plates were then covered and incubated at 4°C overnight. The plates were washed with PBS-T, to remove unadsorbed antigen. After a washing step of three washes for 5 min each with PBS-T, each well was blocked with 5% dry-milk (Nestle, Sweden) PBS-T solution for 1 h at 37°C. The plates were again washed as previously described, followed by the addition of sera samples (100 uL, 1:150 in PBS-T, duplicate wells), and incubated at 37°C for 1 h. After the washing step, monoclonal anti-human IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) were added at an optimized dilution (1:5000) in PBS-T and incubated at 37°C

for 1 hour. Following a final washing step, o-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) was added and the ODs were measured after 15 min as absorbance at 450 nm using an ELISA spectrophotometer (Biocrom EZ Read 400, United Kingdom). The OD readings were blanked with the PBS-T, and the cutoff value was used to discriminate between the positive and negative results. This cutoff value was based on the results of a ROC statistical analysis.

2.5. Western Blotting Assay

Lastly, a Western Blotting assay was used to confirm ELISA close to cut-off absorbance (borderline). The rTES-30 (20 ug/ml) was applied in a 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, USA) by using a transblot apparatus (Bio-Rad, USA) at 4°C overnight. Protein transfer to membrane was checked with Ponceau S staining (Sigma-Aldrich, USA). The membrane was cut into strips and blocked with 5% dry-milk (Nestle, Sweden) PBS-T solution for 1 h. The strips were then incubated with serum/sera samples (diluted 1:200 in PBS-T) at 4°C overnight, followed by monoclonal anti-human IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) were added at an optimized dilution (1:5000) in PBS-T and incubated at 37°C for 1 hour. Between each step, the strips were washed with PBS-T during 5 minutes. Finally, DAB Solution (0.025% 3,3′-Diaminobenzidine, 0.0009% H_2O_2 and 0.05M Tris/HCl-solution, Sigma Aldrich, USA) were used to develop the blots. Statistical analysis was performed by Pearson chi-square, two-way ANOVA and ROC curve in GraphPad software.

2.6. Ethics

The study was approved by the Research Ethics Committee in the Health Area, FURG (protocol no. 102/2012).

3. RESULTS

Protein expression could be detected on SDS-PAGE after 72 hours of induction in methanol, while no band was detected prior to 48 hours. In addition, the intensity of the band did not change progressively post 72 hours.

After concentration with PEG-20,000 (Sigma Aldrich, USA) technique, we could observe a unique band on SDS-PAGE (Figure 1), so an extra purification step was not needed; thus, we obtained 183.5 mg (rTES-30) and 178.5 mg (rTES-120) per liter of culture. Also, after 18 hours of deglycosylation incubation, we managed to obtain a deglycosylated product: SDS-PAGE revealed two bands, one with a higher kDa (60~70, glycosylated) and one with lower kDa (~25, deglycosylated).

As both rTES-30 and rTES-120 had the same apparent molecular mass in SDS-PAGE (Figure 1), we did an extra confirmation step to confirm that they were different proteins. DNA was extracted from the pellet after expression and applied on a PCR with specific primers for both genes; we observed two different bands that corresponded to the proteins, one with 641 base pairs (rTES-30) and one band with 506 base pairs (rTES-120).

After confirmation of the proteins, we tested their capacities to diagnose antibodies against *Toxocara* spp. in sera previously reported as seropositive with *Toxocara* spp., as shown in Table 1. Both proteins had the same capacity to identify positive samples ($p=0.2769$). In addition, glycosylation did not appear to influence the diagnostic performance in rTES-30 ($p>0.9999$) nor rTES-120 ($p>0.9999$).

Table 1. Optimum sensitivity and specificity for each protein in samples without co-infection after ROC analysis.

Protein	Positive Samples (Sensitivity)	Negative Samples (Specificity)
rTES-30	25/45 (55.6%)	43/45 (95.6%)
rTES-30 Deglycosylated	26/45 (57.8%)	43/45 (95.6%)
rTES-120	31/45 (68.9%)	43/45 (95.6%)
rTES-120 Deglycosylated	31/45 (68.9%)	44/45 (97.8%)

To confirm the positive results from ELISA, we performed a Western Blotting assay using a pool of *Toxocara* spp. positive sera. In Figure 2, we show the bands of rTES-30 and rTES-120, as well as their deglycosylated product being recognized by a patient's antibody. As a positive control, we used a previously-studied rTES-120 produced in *E. coli*.

In Table 2, we verified the specificity of the proteins against other parasites. No difference was observed between the two proteins (p=0.2059); however, there was a significant difference in specificity (p=0,0498) between the recombinant proteins and native TES (data not shown), the standard tool for diagnosis of *T. canis* infection.

Table 2. Cross-reactivity of recombinant proteins against parasites with cross-reactivity potential to *Toxocara* spp. infection.

	Cross-Reactivity[a]					
Protein	*Ascaris lumbricoides*	*Trichuris trichiura*	*Ancylostomids*	*Strongyloides stercoralis*	*Hymenolopis nana*	*Fasciola hepatica*
rTES-30	0/9 0%	0/2 0%	0/4 0%	0/16 0%	0/6 0%	0/3 0%
rTES-120	3/9 33.33%	0/2 0%	0/4 0%	1/16 6.25%	0/6 0%	0/3 0%

a) Cut-off calculated by ROC curve assay of 40 *Toxocara* spp. negative patients

4. DISCUSSION

In this study, we expressed rTES-30 and rTES-120 in *Pichia pastoris* and tested two recombinant proteins to be used in *T. canis* infection diagnostic. Prior to this study, only one other study has investigated the recombinant TES-120 produced in *P. pastoris* application to immune diagnostic of *T. canis* infection [8]. However, the study focused on the specificity of rTES-120, tested by immunoblot assay, and the topic of sensitivity was not investigated.

In the other hand, recombinant TES proteins in *E. coli* have been assayed previously by our group and we showed a high specificity (92.5-95%) and sensitivity (75.6-84.4%)[11], however *E. coli* expression presented a cytoplasmic protein expression that was insoluble, while *P. pastoris* have the advantage of secreting the target protein to

cell exterior, being soluble[3,6,12,13]. As *E. coli* insoluble protein could have sensitivity issues due to denaturing requirements to solubilize it; expressing in yeast could be a valid alternative to avoid protein refolding. In this study, we present recombinant TES proteins that were successfully secreted to the medium, at an adequate level of purity, unenquiring further chromatography affinity treatment.

To verify the sensitivity and specificity of recombinant antigens produced by *P. pastoris*, we used the same tools recommended for the diagnosis of *T. canis* (ELISA and Western Blotting)[2]. While specificity was close to 100%, the sensitivity of the recombinant proteins (56%~69%) was not as high as proteins produced in *E. coli* (80~93%), as reported by Mohamad et al.[3] and our previous results (75.6–84.4%). It is important to emphasize that the same ELISA protocol and serum panel was used for both the *E. coli* and *P. pastoris* proteins.

Regarding specificity against other parasites, we present a product with high specificity (95.6%), complementing the previous study by Fong and Lau [8], which evaluated specificity against cysticercosis, filariasis, malaria, amebiasis. and toxoplasmosis. In the present study, we complemented the specificity of recombinant proteins against ascariasis, ancylostomiasis, strongyloidiasis, hymenolepiasis, and fasciolosis; these four diseases affect populations of developing countries and, because of their cross-reactivity, they have potential to influence the diagnosis of *T. canis* infection based on native TES[1,14,15].

Aside from the protein secretion mechanism, one primary difference between the two expression systems is the glycosylation capacity of *P. pastoris.* We chose to investigate whether deglycosylation could increase sensitivity[6,12], but we found no statistical difference ($p>0.05$) in sensitivity nor specificity, suggesting that the mannose products did not influence the performance of the proteins.

It is important to state that *P. pastoris* glycosylation differs from glycosylation in humans so we did not expect that the proteins produced would have the same reactivity. Specifically, *P. pastoris* provides a hyper-mannosylation (N- and O-linked glycosylation), a simple mechanism compared to the diversity in the glycan structure assembled in the Golgi cisternae of mammals[16]. For example, Roldan et al. [17] reported an increase in sensitivity after deglycosylation of native TES proteins, showing that native TES does have a different glycosylation mechanism than *P. pastoris*.

Our main limitation to testing the deglycosylation hypothesis was that we did not manage to obtain a pure deglycosylated product, as we could still observe a glycosylated band on the SDS-PAGE (Figure 1). Nevertheless, considering that we applied a mixture of proteins in each test and that deglycosylated products were at a higher concentration (evidenced by a more intense band in SDS-PAGE, Figure 1), we should expect an increase or decrease in sensitivity, even with an “impure” product, as deglycosylated and glycosylated products should coat the microplate simultaneously[3,7]. Also, our deglycosylation methodology did not remove the O-glycosylation sites produced by *Pichia pastoris*, as the bands in Figure 2 were not at the same height (after deglycosylation) as the *E. coli* rTES-120 band; the presence of O-glycosylation could account for the difference between the sensitivities of *E. coli* and *Pichia pastoris* products, as suggested by Elefant et al.[18], Li et al.[19], and Irani et al.[20] studies, which collectively showed that glycosylation reduces sensitivity in serodiagnosis.

5. CONCLUSIONS

In conclusion, the sensitivity in the serodiagnosis diminished any advantage that *P. pastoris* expression could have. While, we do not recommend *P. pastoris* recombinant TES production as an alternative for the diagnosis of toxocariasis, we evidencied the necessity of studies about the *P. pastoris* recombinant proteins assessing their glycosylation and protein folding.

6. ACKNOWLEDGEMENTS

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v. Figure Legends

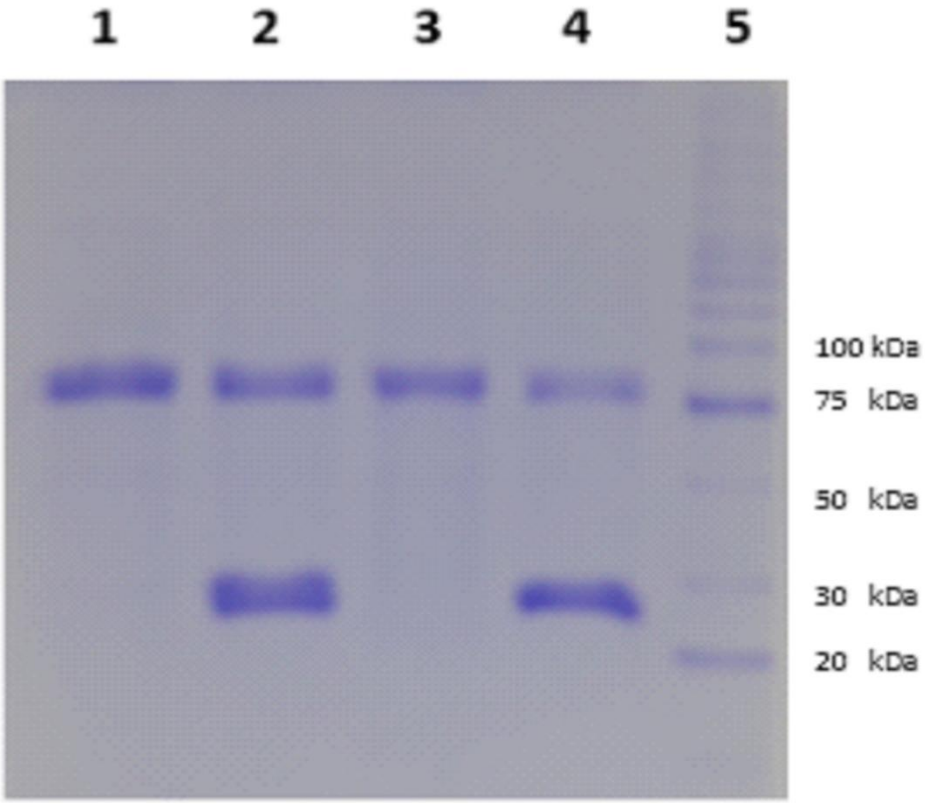
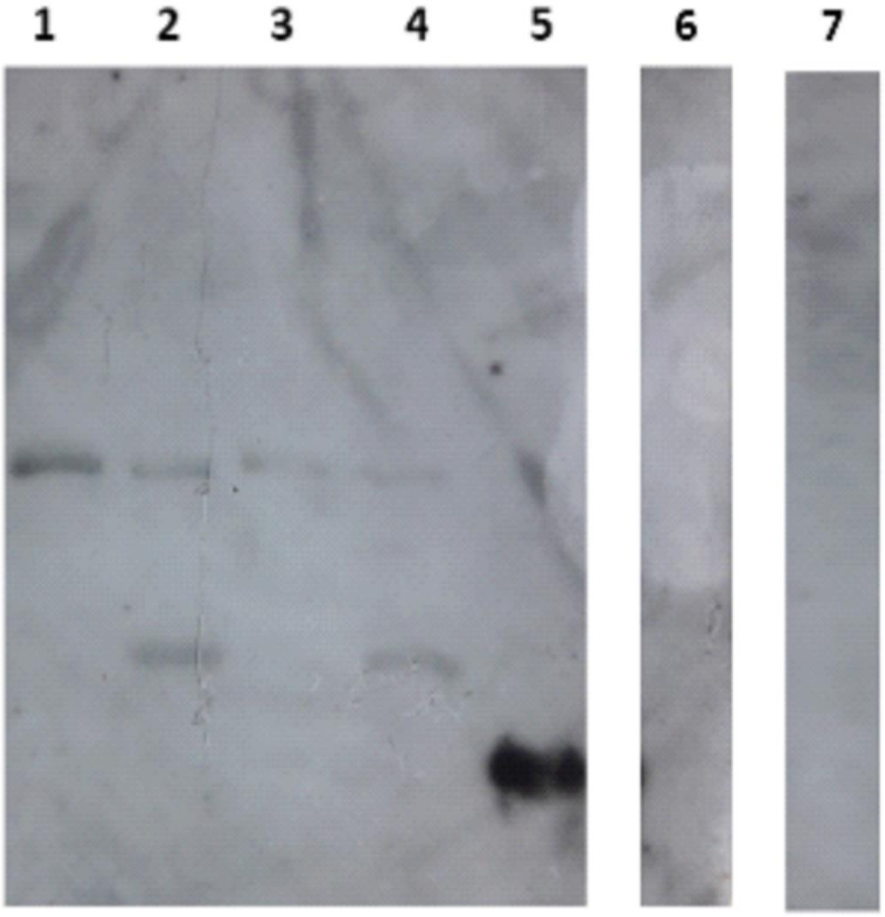


Figure 1. Evaluation of concentration and deglycosylation of the recombinant proteins rTES-30 and rTES-120 on 12% SDS-PAGE stained with Comassie Brilliant Blue R-25. Lane 1: rTES-30; 2: rTES-30 deglycosylated; 3: rTES-120; 4: rTES-120 deglycosylated; 5: BenchMark™ Protein Standard (Thermo Fischer Scientific, USA)



1702

1703 Figure 2. Performance of the recombinant antigens on a pool (n=4) of positive
1704 and negative-Toxocara spp. sera on chemiluminescent Western Blotting. Lane
1705 1: rTES-30 with positive sera; 2: rTES-30 deglycosylated with positive sera; 3:
1706 rTES-120 with positive sera; 4: rTES-120 deglycosylated with positive sera; 5:
1707 positive control (Escherichia coli recombinant TES-120, 24 kDa) with positive
1708 sera; 6: rTES-30 with negative sera; 7: rTES-120 with negative sera

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4.4 Artigo 3 – The serodiagnostic potential of recombinant proteins TES–30 and TES–120 in an indirect ELISA in the diagnosis of toxocariasis in cattle, horses, and sheep

Artigo publicado na revista *Plos One*

The serodiagnostic potential of recombinant proteins TES–30 and TES–120 in an indirect ELISA in the diagnosis of toxocariasis in cattle, horses, and sheep

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Abstract

Toxocariasis is a zoonotic disease that affects humans and animals alike. Although recombinant proteins are widely used for its diagnosis in humans, their performance in companion and production animals remains unknown. This study aimed to investigate the serodiagnostic potential of the recombinant proteins rTES–30 and rTES–120 from *Toxocara canis* in an indirect ELISA for cattle, horses, and sheep. Serum samples collected from the animals were tested with indirect ELISA and Western Blotting using *T. canis* TES–30 and TES–120 recombinant proteins produced in *Escherichia coli*, as well as native-TES. In the ELISA, rTES–30 showed high serodiagnostic potential in sheep and horses (92.6% and 85.2%, respectively), while the sensitivity of rTES–120 was higher in cattle and horses (97.2% and 92.6%, respectively). Furthermore, a highly positive association was observed between native and recombinant proteins in seropositive samples, while a moderately positive association was observed in seronegative samples, probably due to the lower specificity of native TES. In conclusion, our study indicates that the use of recombinant proteins in an indirect ELISA is an effective tool for the serodiagnosis of toxocariasis in animals, with the choice of protein being species-dependent.

Introduction

Endoparasites are among the important threats to the health of companion and production animals, whereby infections diminish the economic value of the animals [1]. Among infections caused by endoparasites, toxocariasis is important. It is

transmitted through the infectious eggs of *Toxocara* sp., and its symptomatology varies depending on the larval migration and parasite and host species. For example, *Toxocara vitulorum* infection in ruminants was detected in 21.1% out of 819 animals in Qinghai Tibetan Plateau, China, and was associated with increased morbidity and mortality, causing important economic loss to the farmers [2,3].

Companion and production animals such as horses and sheep, that commonly share ambient surroundings with definitive hosts (canids and cattle), are prone to become paratenic hosts of the parasite, acting as vectors in the spread of the parasite to a vast number of species and adversely affecting the economy of the region [4,5].

The clinical diagnosis of toxocariasis is difficult as most manifestations are non-specific [6]. Therefore, the only viable diagnostic options are laboratory-based and depend on whether the host is definitive or paratenic, whereby fecal examination and enzyme-linked immunosorbent assay (ELISA) in combination with western blotting (WB) are used [2,6]. The fecal examination is time-consuming and requires expertise to produce a viable diagnosis. Furthermore, depending on the degree of infection, it has low sensitivity, producing more false negatives than serological methods [7,8].

In contrast, ELISA and WB are indirect assays, but are rapid, accessible, and require minimum training [9]. However, the *Toxocara* excretion-secretion protein family (TES), obtained from the larval culture, is required for these assays, which is a laborious process and also presents a number of cross-species reactions, especially with other helminths commonly found in livestock, resulting in an erroneous diagnosis [10–12]. In order to find an alternative, several studies have investigated the potential of recombinant proteins for the diagnosis of toxocariasis in an attempt to reduce the time, cost, and cross-reactivity against native TES [13–16].

This study investigates the serodiagnostic potential of *T. canis* TES–30 and TES–120 recombinant proteins in an indirect ELISA for the detection of toxocariasis in animals. This technique could represent an important breakthrough in increasing the specificity of serodiagnosis and facilitating a rapid and precise diagnosis

Materials and Methods

Cloning and Expression of Recombinant Proteins

The recombinant proteins were cloned, expressed and purified as described in our previous studies [17-18].

Production of native TES

Adult worms of *T. canis* were obtained by treatment of young (4–8 weeks-old) dogs with 15 mg/kg pyrantel pamoate. The female parasites were subjected to hysterectomy to obtain parasite eggs, which were incubated for 28 days in 2% formalin at 28 °C to allow the formation of embryos [19].

The native TES antigens were prepared as described by Savigny (1979) [20]. In summary, the larvae of *T. canis* were grown in RPMI 1640, and the medium was collected every three days, pooled, and centrifuged. The supernatant was filtered through a 0.2 µm filter (Sigma Aldrich, USA) into a dialysis tube (molecular weight cut-off of 6.000–8.000 Da; Sigma Aldrich, USA). The solution was dialyzed against 250 volumes of distilled water at 4 °C. After dialysis, the supernatant was concentrated in a vacuum concentrator, reconstituted in distilled water, and stored in aliquots at −70 °C. The proteins were further quantified using a Pierce BCA kit (Thermo Fisher Scientific, USA).

Sample Collection

The samples were collected from three sera panels from all randomly selected male and female animals aged more than one year. Initial (prior to this study) blood collection was performed from the jugular vein of animals, using a vacutainer tube. The serum samples were stored at –20 °C until use. Sera panel-1 consisted out of 104 non-immunized sheep samples that were randomized from a total of 1,642 samples collected from 95 farms across 21 countries [5]. Sera panel-2 consisted out of samples from 46 non-immunized *Bos taurus* cattle that were collected for the study by Cunha *et al.* (2012) [21]. Sera panel-3 consisted out of samples from 38 non-immunized horses used for the study by Moraes *et al.* (2014) [22].

For the negative controls for serodiagnosis of each animal species, negative sera samples were selected from the sera banks of each species that were maintained by the UFPel Parasitology Laboratory. These samples were collected from animals that tested negative in the excretory–secretory *Toxocara canis* (TES) antigen enzyme-linked immunosorbent assay (ELISA) and were born in farms where there were no dogs. Fetal sera were also used as negative controls. Positive sera (positive controls) were collected from two adult animals of each species following experimental vaccination with rTES–30 and rTES–120 (400 ng) by the subcutaneous application.

Western Blotting (WB) Assay

The WB assay was used in two phases during the study. First, it was used to test the samples in order to distinguish negative sera from positive sera. Secondly, it was used to confirm ELISA results close to the cut-off absorbance (borderline).

The following method was followed in both the WB assays: 20 µg/mL each of rTES–30 and rTES–120 were electrophoresed on a 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, USA) using a transblot apparatus (Bio-Rad, USA) overnight at 4 °C. The transfer of proteins to the membrane was confirmed using Ponceau S staining (Sigma-Aldrich, USA). The membrane was cut into strips and blocked using 5% dry-milk (Nestle, Sweden) in PBS-T for 1 h. The strips were then incubated with sera samples (diluted 1:200 in PBS-T) overnight at 4 °C, followed by incubation with anti-horse IgG (whole molecule) horseradish peroxidase (Sigma Aldrich, USA), anti-sheep IgG (whole molecule) horseradish peroxidase (Sigma Aldrich, USA), or anti-bovine IgG (whole molecule) horseradish peroxidase (Sigma Aldrich, USA) at an optimized dilution (1:10000, 1:5000, or 1:2500, respectively) in PBS-T, and incubated for 3 h at room temperature. The strips were washed with PBS-T for 5 min each between each step. Finally, DAB Solution (0.025% 3,3′-diaminobenzidine, 0.0009% H_2O_2, and 0.05 M Tris/HCl-solution, Sigma Aldrich, USA) was used to develop the blots.

Indirect ELISA

ELISA was used to verify the antigenicity of the proteins. The ELISA protocol was optimized prior to the study. Each well of the 96-well flat-bottomed microtiter plate (Nunc Immuno Maxisorp, Thermo Fisher Scientific, USA) was coated with 100 µL of each antigen at the optimum concentration (50 ng) in 0.02 M bicarbonate buffer, pH 9.6. The plates were then covered and incubated overnight at 4 °C. The plates were washed with PBS-T to remove unattached antigens. The plates were washed thrice for 5 min each time with PBS-T, and then each well was blocked with 5% dry-milk (Nestle, Sweden) in PBS-T solution for 1 h at 37 °C. The plates were again washed

as previously described, followed by the addition of sera samples (100 µL, 1:150 in PBS-T, duplicate wells) and incubation for 1 h at 37 °C. After the washing step, anti-horse IgG (whole molecule) horseradish peroxidase (Sigma Aldrich, USA), anti-sheep IgG (whole molecule) horseradish peroxidase (Sigma Aldrich, USA), or anti-bovine IgG (whole molecule) horseradish peroxidase (Sigma Aldrich, USA) were added at optimized dilutions (1:10000, 1:5000, 1:2500, respectively) in PBS-T, and incubated for 1 h at 37 °C. After a final washing step, o-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) was added, and after 15 min, the ODs were measured at 450 nm using an ELISA spectrophotometer (Biochrom EZ Read 400, United Kingdom). The OD readings were adjusted with PBS-T as blank, and the cut-off value was used to distinguish between the positive and negative results. These cut-off values were based on the results of Receiver Operating Characteristic (ROC) statistical analysis (S1-S3 Files). The cut-off values were: cattle- 0.332 (rTES–30) and 0.414 (rTES–120); horses- 0.099 (rTES–30) and 0.189 (rTES–120); and sheep- 0.499 (rTES–30) and 0.4015 (rTES–120).

Statistical analysis

The data were statistically analyzed using Pearson's chi-square and Pearson's correlation matrix (for qualitative variables, seropositive and seronegative); two-way ANOVA (for quantitative variables, OD readings); and ROC curve (to determine cut-off values) using the statistical software GraphPad Prism version 7.

The cut-off values were calculated using the method described by Hanley (1982) [23], by plotting sensitivity against specificity. The negative and positive samples were added on the software based on the results from the initial WB. The cut-off for each

animal species was chosen based on the likelihood ratio using the method described by Johnson (2004) [24].

Ethics

This retrospective study was previously approved by the Federal University of Pelotas Ethical Research Committee under protocol CEEA 2133 [5,21,22].

Results and discussion

Yield, size, and purification of the recombinant proteins were as described in our previous studies [17-18]. Similar to Farmer *et al.* (2017) study, these proteins were stored in urea buffer [25].

The sensitivity and specificity of the recombinant proteins in the indirect ELISA are presented in Tables 1–3. There was no benefit associated with the use of both proteins at the same time (p=0.9313), in any of the animal serodiagnostic assays studied.

Table 1. ELISA sensitivity and specificity for each recombinant protein as determined by a Receiver Operating Characteristic (ROC) analysis for cattle.

Parameter	rTES-30	rTES-120
Positive samples (sensitivity)	14/36 (38.89%)	35/36 (97.22%)
Sensitivity 95% confidence interval	23.14% - 56.54%	85.47% - 99.93%
Negative samples (specificity)	9/10 (90.00%)	9/10 (90.00%)
Specificity 95% confidence interval	55.5% - 99.75%	55.5% - 99.75%
Cut-off	> 0.332	> 0.414
p value	< 0.001	

Table 2. ELISA sensitivity and specificity for each recombinant protein as determined by a Receiver Operating Characteristic (ROC) analysis for horses.

Parameter	rTES-30	rTES-120
Positive samples (sensitivity)	23/27 (85.19%)	25/27 (92.59%)
Sensitivity 95% confidence interval	66.27% - 95.81%	75.71% - 99.09%
Negative samples (specificity)	10/11 (90.91%)	10/11 (90.91%)
Specificity 95% confidence interval	58.72% - 99.77%	58.72% - 99.77%
Cut-off	> 0.099	> 0.189
p value	0.6687	

Table 3. ELISA sensitivity and specificity for each recombinant protein as determined by a Receiver Operating Characteristic (ROC) analysis for sheep.

Parameter	rTES-30	rTES-120
Positive samples (sensitivity)	25/27 (92.59%)	15/27 (55.56%)
Sensitivity 95% confidence interval	79.7% - 96.92%	35.33% - 74.52%
Negative samples (specificity)	73/77 (94.81%)	76/77 (98.7%)
Specificity 95% confidence interval	90.02% - 97.73%	92.98% - 99.97%
Cut-off	> 0.499	> 0.401
p value	0.0041	

The confirmation of the ELISA cut-off can be seen in Fig 1. Despite the lower sensitivity of the ELISA in case of some recombinant proteins and animal combinations, the WB assay permitted the visualization of both bands in the seropositive sera.

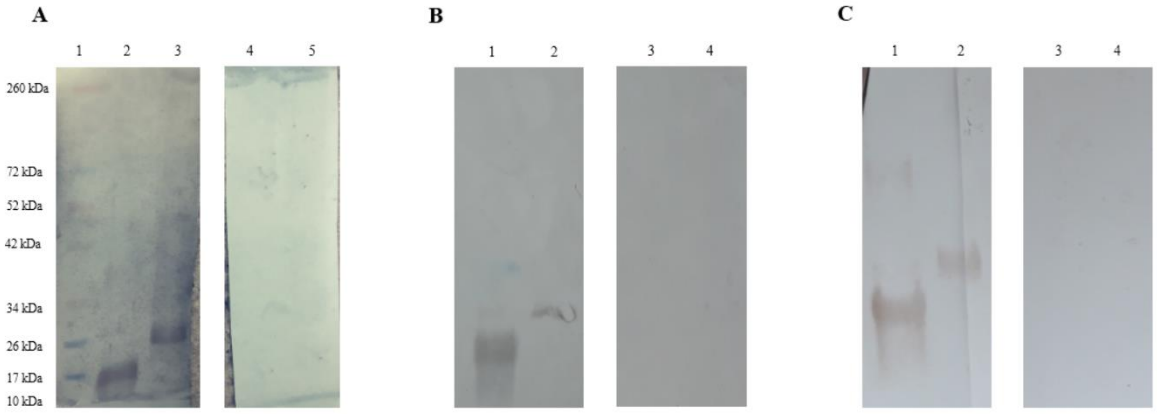

Fig 1. (A) Bovine ELISA close to cut-off confirmation via WB assay. Lane 1: PageRuler™ Pre-stained Protein Ladder (Thermo Fisher Scientific, USA); Lane 2: rTES–30 with pooled sera from four seropositive animals; Lane 3: rTES–120 with pooled sera from four seropositive animals; Lane 4: rTES–30 with pooled sera from four seronegative animals; Lane 5: rTES–120 with pooled sera from four seronegative animals. **(B) Horse ELISA close to cut-off, confirmed by the WB assay.** Lane 1: rTES–30 with pooled sera from four seropositive animals; Lane 2: rTES–120 with pooled sera from four seropositive animals; Lane 3: rTES–30 with pooled sera from four seronegative animals; Lane 4: rTES–120 with sera from four seronegative animals; **(C) Sheep ELISA close to cut-off, confirmed by the WB assay.** Lane 1: rTES–30 with pooled sera from four seropositive animals; Lane 2: rTES–120 with pooled sera from four seropositive animals; Lane 3: rTES–30 with pooled sera from four seronegative animals; Lane 4: rTES–120 with pooled sera from four seronegative animals.

In this study, we assessed the performance of a proposed alternative (recombinant TES in an indirect ELISA) method for the serodiagnosis of toxocariasis. Our methodology for the serodiagnosis of toxocariasis was based on our previous study

[18] and that of Nguyen *et al.* [26,27] study. In both the studies, TES–30 (in recombinant and native forms) was demonstrated to be the most specific biomarker for the serodiagnosis of toxocariasis in paratenic animals and humans [18,27]. Although we had evaluated different species in the earlier study and this study has employed recombinant proteins for the serodiagnosis of toxocariasis in production animals for the first time, we have applied the best available tool for the serodiagnosis of unknown samples, in addition to positive and negative controls.

One of the major problems with the diagnosis of toxocariasis using native TES is the lack of specificity, because of the presence of other families of helminths that are common parasites in production animals [10,11,28]. In humans, the diagnosis of toxocariasis not only requires a diagnostic laboratory but also epidemiological and clinical data to avoid misdiagnosis, which are unavailable in production animals [9]. To overcome this obstacle, we employed an extra method to avoid false positives and false negatives, wherein we assayed each sample with recombinant proteins in a blotting assay instead of using ELISA as the starting point. This extra step permitted the confirmation of seronegatives, making the ELISA cut-off viable. Finally, only samples that were above the cut-off with one recombinant protein, as well as native TES, were considered seropositive, thus nullifying the possible low sensitivity of recombinant proteins and the low specificity of native TES altogether.

Overall, we observed a high specificity with the recombinant proteins, concordant with the study of Mohamad *et al.* [13]. In addition, these proteins (rTES–30 and rTES–120) had been previously tested against ascariasis, trichuriasis, ancylostomids, strongyloidiasis, hymenolepiasis, and fasciolosis in humans without any cross-reactions [17]. However, the production animals are in close contact with the environment and human specificity should not be extrapolated to animal specificity, as

different parasites could introduce new specificity issues. The issue of specificity is yet not to be completely explored and should be studied further [29,30,31].

It is worth noting that we chose not to adsorb the sera with an *Ascaris suum* antigen in an attempt to achieve a better specificity in native TES [11]. We made the decision to compare the absolute efficacy of recombinant against native TES because adding another antigen to the procedure in the disease diagnosis of production animals would have increased the diagnosis-related cost further, which is an important factor for farmers [31]. Moreover, the adsorption of *Ascaris suum* antigens only prevents cross-reactions with the *Ascarididae* family; hence, cross-reactivity issues with fascioliasis and strongyloidiasis infections would remain [32,33].

ELISA sensitivities with recombinant proteins in different animal species were highly variable. In horses, ELISA with recombinant proteins had a high sensitivity, while rTES–30 ELISA was more sensitive in sheep and rTES–120 ELISA was better for bovine serodiagnosis. As the recombinant proteins are significantly different in amino acids structure, we suggest that the immune systems of different species react differently to each protein, perhaps due to the differences in pathogeny and proteomic profile that the *Toxocara* larvae present in the physiological and immunological functions of each animal species [34]. For example, our previous study reported that rTES–30 appeared to be the only viable tool for serodiagnosis in mice [18].

rTES–30 has been widely used as a specific biomarker for toxocariasis in paratenic hosts. This is in agreement with our results, where rTES–30 showed a better sensitivity in both sheep and horse [25,27]. rTES-30 ELISA has a high sensitivity towards *T. canis* and *T. cati* infections in the paratenic hosts, being capable of the serodiagnosis of both parasites, although sensitivity against *T. vitulorum* and *T. malaysiensis* remains unknown [35,36].

On the other hand, rTES–120 performed better with cattle, which could be influenced by the fact that cattle are definitive hosts of *T. vitulorum*. Therefore, rTES–120 could potentially be related to the full development of the larvae; however, more studies are required to support this conclusion [14,35,36].

In this study, we could not confirm the actual disease, because we did not conduct biopsies on the paratenic hosts or fecal examinations on the definitive hosts. As toxocariasis is a chronic disease with active and dormant larvae phases, infected animals could be misdiagnosed as false negatives in biopsies or fecal examination which are assays that have lower sensitivity in this disease [8]. In these cases, WB with TES–30 has been shown to be the most sensitive and specific method for this purpose [27]. Nevertheless, the main objective of this study was to assess a potentially rapid, feasible, and inexpensive tool for the serodiagnosis of toxocariasis for an entire farm animal population, for which we compared the efficacy of our methods against the standard method (ELISA TES).

Conclusions

In conclusion, our study indicates that it is beneficial to use recombinant proteins as a substitute for the laborious native-TES. However, selection of the recombinant protein to be used may depend on the animal species being diagnosed.

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Supporting Information

S1 File. ELISA and cut-off values for native and recombinant TES in cattle. File containing the enzyme-linked immunosorbent assay absorbances, the receiver operating characteristic analysis and the resulting cut-offs for toxocariasis diagnosis using native and recombinant *Toxocara* excretion-secretion proteins in cattle.

S2 File. ELISA and cut-off values for native and recombinant TES in sheep. File containing the enzyme-linked immunosorbent assay absorbances, the receiver operating characteristic analysis and the resulting cut-offs for toxocariasis diagnosis using native and recombinant *Toxocara* excretion-secretion proteins in sheep.

S3 File. ELISA and cut-off values for native and recombinant TES in horses. File containing the enzyme-linked immunosorbent assay absorbances, the receiver operating characteristic analysis and the resulting cut-offs for toxocariasis diagnosis using native and recombinant *Toxocara* excretion-secretion proteins in horses.

4.5 Manuscrito 2 – Development of a lateral flow test for toxocariasis serodiagnosis using TES-30 recombinant antigen.

Manuscrito a ser submetido à revista *Diagnostic Microbiology and Infectious Disease*

Development of a lateral flow test for toxocariasis serodiagnosis using TES-30 recombinant antigen

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Abstract

Point-of-care tests are essential for control of neglected diseases such as toxocariasis. While recombinant proteins introduced an economic and viable way to serodiagnose toxocariasis, a point-of-care could enhance early diagnosis potential, raising the chances of cure of the treatment. This study aims to develop a lateral flow assay with the diagnostic potential of *Toxocara* spp. in children. Thirty eight serum samples were collected from children seropositive and seronegative to *Toxocara* spp. These samples were tested in a lateral flow assay using *T. canis* TES-30 recombinant protein produced in *Escherichia coli*. Concordance with ELISA rTES-30 and Western Blotting was presented in 73.9% of positive serums and 73.3% of negative serums. Further blotting confirmed the false-positives and false-negatives of the point-of-care test. In conclusion, our study presents a point-of-care alternative for toxocariasis serodiagnosis.

Introduction

While infection by *Toxocara* spp*.* have been discovered in the middle of the XX century, 1950 to be exact, up until to this date it is considered a neglected disease (1).

The most aggravating pathology of toxocariasis occurs when the parasite invade the central nervous system and reachs the eyes of the patient, with possible outcomes of blindness and severe sequelae (2,3).

To complicate the situation, it is common for patients acquire the infection during the childhood (4), while the mortality is low (3), any parasite damage done can affect for the remaining of the patient life, as the treatment efficacy can vary from 40-75% (5). To the treatment be more successful, an early and precise diagnosis must be applied (5).

The diagnosis of toxocariasis are based on clinical, epidemiological and laboratory exams (1,6). As the symptoms are unspecific, the most precise method to diagnose an infection of *Toxocara* spp. is combining the epidemiology of the region with the risk factors of the patients (contact with young dogs) and the laboratory exams (1,6). The laboratory method still used up until this date is the ELISA and Western Blotting with native *Toxocara* excretory secretory antigens (TES), however it is laboriously produced *in vitro* and presents cross-reactions with others helminths, common in children (1,6). As an alternative, recombinant proteins, specially rTES-30, have been used in research presenting good sensitivity (81.8%) and specificity (94%) in children (7).

While ELISA with rTES-30 appears to be a good technique for diagnosis, it still requires laboratory expertise to run such exams (8). As a solution, reducing the time required to run the exam and the necessity of laboratory expertise to analyze the results, point-of-care tests could be used (9,10). The advantage of this methodology is that the tests present a good sensibility and specificity, almost immediate results and does not require much training to perform the test (9,10).

Therefore, the aim of this study is to present a point-of-care alternative for toxocariasis: a lateral flow test with rTES-30 for serodiagnose *Toxocara* spp. infection; and evaluate its sensitivity and specificity against the rTES-30 ELISA.

Materials and Methods

Cloning and Expression of Recombinant Protein

The cloning and expression of recombinant protein was already been described in Santos *et al* (2018) study (7). Briefly, the synthetic gene of TES-30 (Genbank access 4586556) was cloned in the expression vector pAE, which was used to transform an *Escherichia coli* BL21 DE3 to produce the recombinant protein after inducing with isopropyl β-D-1-thiogalactopyranoside. After cell lysis, the inclusion bodies were ressuspended using 6 M urea phosphate buffer. We purified with a ÄKTA Start and HisTrap HP purification columns (GE Healthcare Life Sciences, USA), using 6 M urea phosphate buffer. After, the protein solution was dialyzed (14,000 MW, Cial, Brazil) in phosphate buffer pH 7,4 and stored at 4 ° C until use.

Sample Collection

Samples were collected from the sera panel of Cadore *et al* study (11). This sera panel contained a total of 23 sera from children previously diagnosed for *Toxocara* spp. infection and 127 sera from children previously diagnosed as not infected by *Toxocara* spp.; of which, all the 23 positive sera were collected and 15 negative-sera were collected. The sera was stored at –20 ° C until use. Positives and negatives samples were diagnosed by clinic data, eosinophilia, seropositivity to *Toxocara* spp.

(via native TES ELISA, rTES-30 ELISA and Western blotting confirmation) and two epidemiological factors: contact with young dogs/cats, and geophagy.

Indirect ELISA and Western Blotting Assay

The indirect ELISA and Western Blotting assay were performed as in Cadore *et al* (2016) and Santos *et al* (2018) studies (7,11). Briefly, native TES was produced in *in vitro* following de Savigny methodology (12) and recombinant TES-30 was produced in *Escherichia coli* as described before (7). The protein at a concentration of 50 ng/well, were coated in a 96-well flat-bottomed microtiter plate (Nunc Immuno Maxisorp, Thermo Fischer Scientific, USA) or transferred, after SDS-PAGE electrophoresis, to a nitrocellulose membrane. After, blocking with 5% dry-milk in phosphate buffer was performed; sera were added at 1:150 dilution in phosphate buffer (added 0.05% tween, PBS-T); and monoclonal anti-human IgG-horseradish peroxidase (Thermo Fisher Scientific, USA) were added 1:5000 dilution in PBS-T; finally, o-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) was added and the optical density (OD) were measured after 15 min at 450 nm using an ELISA spectrophotometer. The OD readings were blanked with the PBS-T, and the cutoff value was used to discriminate between the positive and negative results. This cutoff value was based on the results of a ROC statistical analysis: 0.3415 for rTES-30 (7). Intermediate Zone was considered 20% of the cutoff: 0,2732-0,4098.

In case of native TES, the patient sera were pre-adsorbed *Ascaris suum* antigens, prior to ELISA and Western Blotting assay, to reduce cross-reactions (11).

Lateral Flow Assembly and Testing

Colloidal gold nanoparticles 40 mM at an optical density (OD) of 0.3 were conjugated to polyclonal goat anti-human IgG using the patent protocol IPTSP-UFG. A nitrocellulose membrane card and cellulose fiber absorbent pad (Millipore, USA) were used to prepare the lateral flow membrane. The absorbent pad was placed on the sticky part of the nitrocellulose membrane with an approximate 2 mm overlap between the pad and the nitrocellulose membrane surface. The assembled card was then cut into strips (5 mm width) using a strip cutter (A-Point Technologies Inc., USA). A 0.875 µL volume of purified rTES-30 (0.25; 0.5; and 1 ug/uL) was dotted onto the middle of each strip, and for the control zone, protein A (Sigma Aldrich, USA, 1ug/uL) was dotted at the end of each strip. the strips were dried in a 37 ° C incubator for 3 hours, blocked with a bovine serum albumin solution (Sigma Aldrich, USA), and then placed overnight in a 37 ° C incubator. The next day, the strips were transferred to a dry cabinet (< 20% humidity) at room temperature for storage.

Prior to the evaluation of the sensitivity of the prototype, multiple sample dilutions were applied, reaching the optimal dilution of 25 microliters of each serum sample diluted (1:4) in Trizma® buffer (Sigma Aldrich, USA) placed in the sample pad. The result was read once 10 minutes passed after the sample was applied. The test was interpreted as positive when a red colored line appeared in the test line region. If the test line region remained clear (with no red colored dot seen), the test was considered negative. To validate both positive and negative results, the control with protein A must appear as a red colored line.

Ethics

This retrospective study was previously approved by the Research Ethics Committee of the Federal University of Rio Grande (FURG) (CEPAS 205/2011) (11).

Results and discussion

For this prototype, a total of 10 L of *E. coli* was cultivated to produce rTES-30, yielding purified and dialyzed concentrations of 1.6 mg/L. 10 of each tests (0,25, 0,5 and 1 ug/uL) were tested against the highest O.D (0,6275) on rTES ELISA. From the prototypes, only the prototype with 1 ug/uL of rTES-30 (LF-rT30) showed a good positive line on the trial (Figure 1). With the most promising prototype, we produced 120 strips to evaluate its performance.

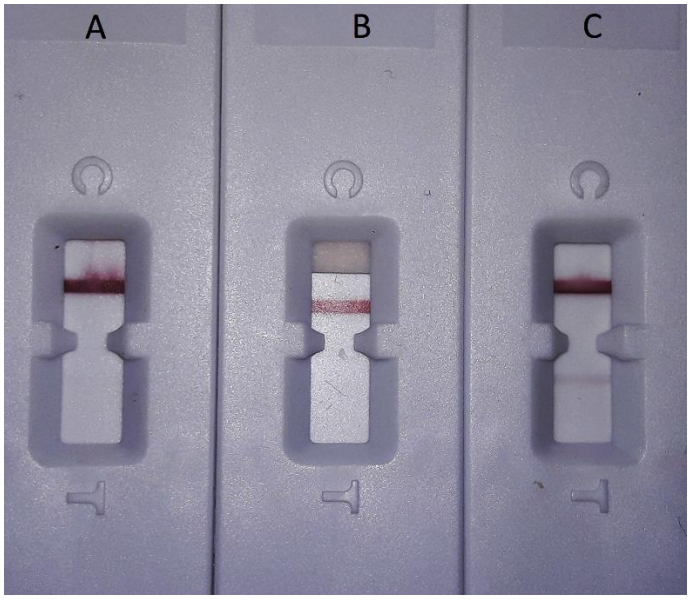

Fig 1. Comparison of 0.25, 0.5 and 1 ug/uL lateral flow prototype with the highest ELISA optical density. A) rTES-30 applied at a concentration of 0.25 ug/uL. B) rTES-30 applied at a concentration of 0.5 ug/uL. C) rTES-30 applied at a concentration of 1 ug/uL.

The sensitivity and specificity of the prototype LF-rT30 was 17/23 (73.9%) and 11/15 (73,3%), respectively, against ELISA rTES and Western Blotting. Blotting of LF-rT30

false-positive samples and false-negative samples have confirmed their diagnosis (7). The entire available prototypes using rTES-30 for point-of-care tests for serodiagnose toxocariasis are presented in Table 1.

Table 1. Sensitivity and specificity of prototypes point-of-care tests using the recombinant TES-30 to serodiagnose *Toxocara* spp. infection.

Prototype/Study	Sensitivity		Specificity
LF-rT30	17/23 (73.9%)		11/15 (73.3%)
Sensitivity *p* value[a]		1.0	
Specificity *p* value[a]		1.0	
Yunus *et al* (2018)	12/29 (51.7%)		50/50 (100%)
Sensitivity *p* value[a]		0.038928	
Specificity *p* value[a]		0.009542	
Lim *et al* (2015)	36/45 (85.7%)		82/88 (90.1%)
Sensitivity *p* value[a]		0.001645	
Specificity *p* value[a]		0.053836	

[a]chi square analysis comparing the referred study to LF-rT30

This is the first study to analyse a lateral flow test for serodiagnose *Toxocara* spp. infection in children, which are more prone for developing toxocariasis, especially neurotoxocariasis and ocular toxocariasis, which effects can leave sequelae for the rest of their life (2–4). Therefore, this prototype could be applied in seroepidemiology and serology vigilance studies, permitting a fast diagnosis, which increases the chances of success of the treatment (5).

Comparing our prototype to others, in Yunus *et al* (2018) study, it is proposed the use of anti-IgG_4 to serodiagnosis, managing to achieve a sensitivity of 51.7% (15/29) with the rTES-30 (13). While Yunus *et al* (2018) study achieve a higher sensitivity with rTES-120 (72.4%) in adults, we have found that rTES-120 have a lower sensitivity in

children, therefore was not the right tool for our suggested population (7,13). In addition, Yunus *et al* (2018) used commercial kits that knowingly possess innumerous cross-reactions with other parasites (13).

One of the probable causes for the higher sensitivity of LF-rT30 ($p = 0.038928$) and lower specificity ($p = 0.009542$) was our choice to use total anti-IgG instead of anti-IgG_4. We made this choice based on our results, in which we found that total anti-IgG had a sensitivity of 84.4% and anti-IgG_4 22.2%; and on Farmer *et al* (2017) study, in which they used anti-IgG to serodiagnose toxocariasis in the National Health and Nutrition Examination Survey applied in the United States (7,14).

Aside from the lateral flow assay, only one other studies proposed a point-of-care alternative for toxocariasis. In Lim *et al* (2015) study, a immunochromatographic device with TES-30 was suggested, presenting a 85.7% of sensitivity, higher sensitivity then LF-rT30 ($p = 0.001645$), however requires 8-10 hours prior to the test, as the test system is sensible for blood cells from the sample (15). Moreover, in Bojanich *et al* (2012) study, a dot-ELISA is suggested, however the method requires more running time (2-3 hours) and native TES it is utilized, which carries possible specificity issues as discussed before (16).

It is important to highlight that only the highest concentration of antigen showed potential of recognizing seropositive samples, therefore it is still possible to increase the sensitivity of the LF-rT30 if we increase concentration of antigen. Regarding the specificity, we performed a Western Blotting to confirm false-negatives and in the blotting assay no bands were observed, this discrepancy could be related to the change of monoclonal peroxidase conjugated anti-IgG from ELISA to polyclonal anti-IgG from the LF-rT30 (as they are a less expensive reagent), therefore changing to monoclonal anti-IgG gold-conjugate could increase its specificity (17).

The main limitation of LF-rT30 was the quantity necessary of the antigen to impregnate each strip. If we calculate, the antigen costs between ELISA and this lateral flow is aggravating. While 10 patients in the LF-rT30 requires 8.75 ug of rTES-30, the same quantity could be applied in a recombinant ELISA (50 ng per patient) to serodiagnose 175 patients with higher sensitivity and specificity.

However, as toxocariasis is a neglected disease, a lateral flow assay it is still an excellent tool to be used in zones that have low accessibility or economic vulnerable, as an ELISA is expensive to setup, as it requires a number of equipment, and requires laboratory expertise to validate the results (1,8,10). In these circumstances, a point-of-care test are essential to fight this disease.

Conclusions

In conclusion, this study show a potential point-of-care test to serodiagnose toxocariasis and could be utilized in regions for seroepidemiology and sorology vigilance studies, that does not have the availability of ELISA, such as economic vulnerable regions which are affected by neglected diseases.

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5. DISCUSSÃO GERAL

A presente tese elucida os avanços no diagnóstico da toxocaríase através do desenvolvimento e avaliação de um ELISA e um teste imunocromatográfico a partir de dois clones recombinantes contendo plasmídeo para a produção das proteínas de secreção-excreção de *T. canis* (TES-30 e TES-120), tendo em vista a construção de uma alternativa ao TES nativo, tecnologia que poderia contribuir para a diminuição da negligência da doença.

Estudos iniciais com essas proteínas recombinantes realizados pelo nosso grupo de pesquisa demonstraram baixas sensibilidades, conforme Tabela 2, mas alta especificidade. Além disso, grande parte da proteína era perdida na etapa de *refoding* durante a diálise, demonstrando a necessidade de uma investigação sobre a estabilidade da proteína e uma padronização minuciosa do ELISA.

Tabela 2. Valores de sensibilidade e especificidade em ELISA com os antígenos rTES-30, rTES-120 e rTES-30 + rTES-120 de acordo com os melhores pontos de corte pela análise ROC.

Antígeno	**Sensibilidade (%)**	**Especificidade (%)**
rTES-30	27,5 (11/40)	95 (2/40)
rTES-120	50 (20/40)	95 (2/40)
rTES-30 + rTES-120	30 (12/40)	95 (2/40)

Durante a padronização do ELISA, detectamos que a proteína não só tinha a mesma sensibilidade quando desnaturada em ureia, bem como mantinha sua estabilidade durante meses em refrigeração. Para assegurar nossa hipótese, um artigo foi publicado por membros do *Centers for Disease Control and Prevention*, órgão de referência mundial, o qual usou a mesma metodologia (utilização na forma desnaturada) com uma proteína recombinante para sorodiagnosticar toxocaríase na população norte americana (Farmer et al., 2017).

A partir desse achado, padronizamos a quantidade de sensibilização da placa de ELISA, bloqueio, lavagens, quantidade de soro e concentração de anticorpo conjugado. E com o protocolo montado, partimos para a avaliação desse ELISA em parceria com a Universidade Federal do Rio Grande: em modelos experimentais (Artigo 1) o qual vimos que o rTES-30 tinha capacidade de detectar soropositividade

para *Toxocara* spp.; em humanos (Artigo 2), no qual apontamos que o rTES-30 era a melhor opção de diagnóstico em crianças, uma das populações mais importantes para toxocaríase; ainda avaliamos (Manuscrito 1) se a produção de recombinantes em *P. pastoris* tinha alguma vantagem para o imunodiagnóstico, a qual não obtivemos sensibilidade adequada para substituirmos o modelo; e, por fim, avaliamos o potencial de diagnosticar diferentes espécies de animais como bovinos, ovinos e equinos com nosso ELISA, no qual conseguimos sensibilidades adequadas para diagnóstico com as proteínas recombinantes (Artigo 3).

Com um ELISA bem avaliado, partimos para o desenvolvimento de um teste imunocromatográfico em parceria com a Universidade Federal de Goiás, as diferenças do nosso modelo para os demais, seria a utilização de um anticorpo policlonal anti-IgG conjugado a ouro coloidal, metodologia de baixo custo e de ótima sensibilidade conforme nossos estudos em humanos demonstraram.

Ao avaliarmos o teste desenvolvido (Manuscrito 2), percebemos que embora tenhamos alcançado sensibilidades e especificidades semelhantes ao de modelos anteriores a um custo menor, a vantagem do teste imunocromatográfico se diminuía pela quantidade de antígeno necessária para diagnosticar um paciente.

Desta forma, enquanto produzimos um teste rápido sensível e específico para sorodiagnosticar infecção por *Toxocara* spp., ainda há necessidade de melhorar esse protótipo ou a produção de proteína recombinante, de maneira que deixe a metodologia mais viável para produção industrial.

6. CONCLUSÃO GERAL

- Foi aprimorada a produção das proteínas recombinantes rTES-30 e rTES-120 em *E. coli* e *P. pastoris*;

- Padronizou-se um ELISA indireto com as proteínas recombinantes para sorodiagnosticar infecção por *Toxocara* spp. em camundongos, humanos, bovinos, ovinos e equinos;

- Desenvolveu-se um protótipo de teste rápido para o imunodiagnóstico da toxocaríase humana com rTES-30;

Em suma, a presente tese demonstrou avanço no diagnóstico de toxocaríase, apresentando um modelo funcional para diagnóstico convencional (ELISA) e rápido (imunocromatográfico) de sensibilidade e especificidade adequadas.

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