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DISSERTAÇÃO DE MESTRADO

FATORES DE RISCO DE INFECÇÃO POR *TOXOCARA CANIS* E ASSOCIAÇÃO DESTA PARASITOSE COM ASMA E ATOPIA

LÍVIA RIBEIRO MENDONÇA

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Trabalho realizado no Laboratório de Alergia e Acarologia, Departamento de Ciências da Biointeração, Instituto de Ciências da Saúde, Universidade Federal da Bahia, como requisito para obtenção do título de Mestre em Imunologia.

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Folha de aprovaçao

"Um pouco de ciência nos afasta de Deus,

muita, nos aproxima".

Louis Pasteur

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SUMÁRIO

LISTA DE ABREVIATURAS

BAL – lavado bronco-alveolar
CCR3 - “chemokine receptor” 3
Células Th - células T “helper”
ELISA - Enzyme-linked Immunosorbent Assay
FcεRI – receptor da porção Fc de imunoglobulina E tipo I
FcεRII – receptor da porção Fc de imunoglobulina E tipo II
IFN-γ– Interferon gama
IgE - Imunoglobulina da classe E
IgG- Imunoglobulina da classe G
IL - Interleucina
ISAAC - Estudo Internacional de Asma e Alergia na Infância
LMV - *larva migrans visceral*
OVA- ovoalbumina
RANTES - Regulated on Activation, Normal T Expressed and Secreted
T. cati - *Toxocara cati*
TcESLA – T. canis excreted-secreted larval antigen (antígeno excretório/secretório de larvas de *T. canis)*
Th1 – célula T “helper” tipo 1
Th2 – célula T “helper” tipo 2
TLR – receptores do tipo Toll
TPC - teste de puntura cutânea

RESUMO

Introdução: O *Toxocara canis* é um parasito helminto cosmopolita de cães que pode infectar os seres humanos provocando a síndrome da *Larva Migrans Visceral* (LMV). Os sinais clínicos da LMV são muito inespecíficos e seu diagnóstico é realizado através da detecção da IgG anti-*T. canis* por ELISA, utilizando antígeno excretório/secretório das larvas de *T. canis* (TcESLA). **Objetivos:** Investigar possíveis associações entre a infecção por *T. canis* com eosinofilia, IgE total e IgE específica contra aeroalérgenos, teste de punctura cutâneo e asma. Objetivou-se ainda, investigar os fatores de risco associados á aquisição desta infecção. **Métodos:** Os pais ou responsáveis das 1.445 crianças do estudo responderam a um questionário ISAAC fase II adaptado para o português, sobre o histórico de sibilo nos últimos 12 meses das crianças. Em seguida estas foram submetidas ao teste de punctura cutâneo (TPC), coleta de sangue para contagem de células periféricas, cultivo para detecção de citocinas e determinação sorológica da IgE específica contra aeroalérgenos, pelo teste Unicap, IgE total determinada por ELISA e detecção de anticorpos IgG anti-*T. canis* por ELISA, utilizando TcESLA e soros pré-absorvido com antígenos de *Ascaris lumbricoides*. Na análise estatística estimou-se Odds Ratio (OR) e Intervalo de Confiança a 95% (IC 95%) na análise univariada e multivariada com regressão logística e análise politômica ajustada para sexo, idade, escolaridade materna, asma dos pais, mofo, esgotamento sanitário e infecções por *A. lumbricoides* e *Trichuris trichiura*. **Resultados:** 53,7% das crianças eram do sexo masculino, 40,5% tinham idade entre seis e sete anos, 48,3% possuíam mães com segundo grau incompleto e em 70% das casas inspecionadas havia mofo nas paredes. Foi detectada infecção de 14,9% para *A. lumbricoides* e 13,8% para *T. trichiura*. A prevalência da infecção pelo *T. canis* foi de 48,4%; 13,4% das crianças tinham pais alérgicos e 22,4% das crianças forma classificadas como asmáticas. Eosinofilia maior que 4% ocorreu em 74,2% e maior que 10% em 25,4% das crianças; IgE total acima do ponto de corte de 0,2 mg/ml ocorreu em 59,6%, e IgE específica para pelo menos um alérgeno de quatro investigados, nos pontos de cortes de ≥0,35 e ≥0,70 foi de 48,5% e 36,8%, respectivamente. Teste cutâneo positivo para pelo menos um dos sete alérgenos testados foi observado em 30,4% das crianças. Os fatores de risco para infecção por *T. canis* determinados neste estudo foram idade, baixa escolaridade materna, pavimentação da rua e contato com cão e/ou gato. A infecção por *T. canis* foi positivamente associada com eosinofilia tanto à 4% como à 10%, com IgE específica para aeroalérgenos ≥0,35 e ≥0,70, aumento de IL-10 e negativamente associada ao TPC. Não foi observada associação desta infecção e asma atópica e não-atópica. **Conclusão:** A soroprevalência da infecção pelo *T. canis* é alta em nossa população. A associação da infecção e o contato com gato é sugestivo que o TcESLA pode reagir cruzadamente com antígenos de *T. cati*. A relação entre baixa escolaridade materna com maior soroprevalência de *T. canis* suporta o caráter sócio-econômico desta patologia. Embora a infecção por *T. canis* seja um fator de risco para eosinofilia, IgE total e IgE específica para aeroalérgenos a infecção está associada negativamente a hipersensibilidade cutânea imediata e, possivelmente, pode impedir a degranulação de mastócitos seja por competição da IgE anti-*T.canis* com a IgE anti-alérgenos na ligação aos receptores de IgE destas células, ou seja pelo aumento da produção de IL-10 mostrado neste estudo. Isto pode explicar também ausência de associação com asma, ambas, atópica e não atópica.

PALAVRAS-CHAVE: *Toxocara canis*, fatores de risco, eosinofilia, atopia, asma, IL-10.

ABSTRACT

Introduction: Toxocara canis is a cosmopolitan helminth parasite of dogs that can infect humans causing the syndrome of visceral larva migrans (VLM). Clinical signs of LMV are very nonspecific and its diagnosis is established by the detection of IgG anti-*T. canis* by ELISA using excretory/secretory larval *T. canis* antigen (TcESLA). **Objectives:** To investigate possible associations between *T. canis* infection with eosinophilia, total IgE and specific IgE against allergens, skin prick tests and asthma. The objective was also to investigate the risk factors associated with acquiring this infection. **Methods:** Parents or guardians of 1445 children in the study answered a ISAAC phase II questionnaire adapted for portuguese on the history of asthma in children in 12 months. Then they were subjected to skin prick test (SPT), blood sampling for peripheral cell count, culture for cytokine detection and determination of specific IgE in serum against aeroallergens at Unicap test, total IgE determined by ELISA and detection of IgG anti-*T. canis* by ELISA using TcESLA and serum pre-absorbed with antigens of *Ascaris lumbricoides*. Statistical analysis was estimated odds ratio (OR) and confidence interval 95% (95%) in univariate and multivariate polytomous logistic regression adjusted for sex, age, maternal education, parental asthma, mold, sewage health and infection by *A. lumbricoides* and *Trichuris trichura*. **Results:** 53.7% of children were male, 40.5% were aged between six and seven years, 48.3% had mothers with incomplete secondary education and in 70% of inspected homes had mold on the walls. Infection was detected from 14.9% to *A. lumbricoides* and 13.8% for *T. trichura*. The prevalence of *T. canis* infection was 48.4%. 13.4% of the parents were allergic, and 22.4% of children were classified as asthmatic. Eosinophilia greater than 4% occurred in 74.2% and greater than 10% in 25.4% of children, total IgE above the cutoff of 0.2 mg/ml occurred in 59.6%, and specific IgE to at least one allergen four investigated in the cut-off points of ≥ 0.35 and ≥ 0.70 was 48.5% and 36.8% respectively. Positive skin test to at least one of the seven allergens tested was observed in 30.4% of children. Risk factors for *T. canis* infection determined in this study were age, low maternal education, paving the street and connect with dog and/or cat. Infection by *T. canis* was positively associated with eosinophilia both the 4% to 10% as with specific IgE to aeroallergens ≥ 0.35 and ≥ 0.70, an increase of IL-10 and negatively associated with the SPT. There was no association of this infection and atopic and non-atopic asthma. **Conclusion:** The seroprevalence for *T. canis* is high in our population. The association of infection and contact with cats is suggestive that the TcESLA can cross-react with antigens of *T. cati*. The relationship between low maternal education with higher seroprevalence of *T. canis* supports the socio-economic development of this pathology. Although infection by T. canis is a risk factor for eosinophilia, total IgE and specific IgE to aeroallergens it is negatively associated with cutaneous hypersensitivity and may possibly prevent the degranulation of mast cells or by competition of anti-IgE *T.canis* with anti-IgE binding to allergens in IgE receptors of these cells, or by increased production of IL-10 shown in this study. This may also explain lack of association with asthma, both atopic and nonatopic

Key-words: *Toxocara canis*, risk factors, eosinophilia, atopy, asthma, IL-10.

1. INTRODUÇÃO

1.1. Caracterização da infecção por *Toxocara canis* e alguns aspectos epidemiológicos

A toxocaríase é uma enfermidade parasitária que acomete canídeos e felídeos, domésticos e selvagens, causada pelo nematódeo gastrintestinal *Toxocara sp.* A Síndrome da *Larva Migrans Visceral* (LMV) é causada pela infecção acidental do homem, com ovos de *T. canis* e mais raramente de *T. cati* liberados nas fezes dos hospedeiros definitivos (canídeos e felídeos domésticos e selvagens). A ingestão acidental destes ovos pelo homem dá início ao um ciclo incompleto, uma vez que no hospedeiro paratênico (homem) este parasito permanece no estágio larval migrando através da circulação sanguínea podendo estabelecer-se em qualquer órgão (JACOB e OSELKA, 1991).

A LMV segundo Lynch et al (1993), é tão ou mais prevalente do que a ascaridíase em crianças de classe social baixa. Infecção humana por *T. canis* tem sido relatada em todo o mundo (THEODORIDIS et al, 2001), porém a prevalência é maior em regiões tropicais e entre populações de baixa renda (NOORDIN et al, 2005). Estudos sobre soropositividade para anticorpos IgG anti-*T.canis* em Bali foi reportada em 63,2% (CHOMEL et al, 1993), na Malásia em 20% (LOKMAN-HAKIN et al, 1993) e nos Estados Unidos variando de 4,6% a 7,3% (HOTEZ e WILKINS, 2009). Chieffi et al (2009) em um levantamento de trabalhos realizados no Brasil, mostraram prevalências variando de 3,72% até 40% nos estados de São Paulo, Pernambuco, Goiás, Acre, Minas Gerais, Espírito Santo e Mato Grosso do Sul.

Recentemente vários trabalhos demonstram o aumento da prevalência desta infecção, evidenciada principalmente através da presença de anticorpos anti-*T.canis* nas populações

humanas, sendo a presença de cães em casa (CHIODO *et. al*., 2006), principalmente filhotes, (DAMIAN et. al., 2007) o principal fator de risco para esta doença (SOWEMIMO, 2009).

A contaminação ambiental também tem sido apontada como um dos principais fatores de risco para infecção por helmintos com potencial zoonótico em cães. Diversos trabalhos no Brasil (GUIMARÃES *et. al*., 2005; ALMEIDA *et. al*., 2007; CAMPOS-FILHO *et. al*., 2008; TYIO *et. al.,* 2008, CHIEFFI et al, 2009) e no mundo (MIZGAJSKA, 1997; DEVERA *et. al.,* 2008; MARTIN e DEMONTE, 2008) mostram que o solo de áreas públicas como praças, parques, "campings" e praia são importantes focos de transmissão e constituem um risco para o homem.

Em Minas Gerais, foi estudada a contaminação do solo por ovos de *Toxocara sp.* em 39 praças públicas e o resultado mostrou que 23,07% das amostras de solo estavam contaminadas com ovos do parasito (COSTA-CRUZ, NUNES e BUSO, 1994). Em Salvador, Bahia, Alcantara-Neves et al (1989) e Santos et al (2006) encontraram 24,8% e 29,24% respectivamante. Estes trabalhos demonstram, portanto, que a presença do cão, a idade do cão e do indivíduo e a contaminação ambiental são importantes fatores de risco para a infecção por *T. canis*. Isto mostra mais uma vez que estudos mais profundos a cerca da epidemiologia da doença precisam ser realizados, uma vez que rotas ainda não investigadas podem ser tão ou mais importantes do que as conhecidas atualmente.

1.2. Asma e outras doenças alérgicas

As doenças alérgicas atingem milhões de pessoas em todo o mundo e um crescimento acelerado se deu nas últimas três décadas (HOLGATE, 1999). Diversos estudos estão sendo

conduzidos na tentativa de buscar possíveis explicações para o aumento súbito de doenças imunomediadas (GALE, 2002; WARNER, 2004). O mais abrangente estudo conduzido até hoje, International Study of Asthma and Allergies in Childhood (ISAAC, 1998), avaliou a prevalência de sintomas de asma, rinoconjutivite alérgica e eczema atópico em 56 países. Um dos achados mais importantes deste estudo foi a observação de que alguns países em desenvolvimento apresentaram prevalências de alergias comparáveis a países desenvolvidos, como o Brasil que ficou entre os dez países com maior prevalência de asma. Em Salvador, estudos do nosso grupo (BAQUEIRO et al., 2007) e outros (MEDEIROS et al., 2000; SOLÉ et al., 2004) demonstraram que as doenças alérgicas ocorrem em mais de 30% da população de baixa renda, e em segmentos da população de alta renda esses números chegam a 44%. Estes achados levaram a mudanças na concepção de que as alergias afetam principalmente os países ricos e trouxe à luz outras hipóteses para o avanço deste fenômeno (HOLGATE, 1999).

Uma das principais hipóteses tentando explicar o aumento súbito destas doenças foi proposta por Strachan (1989) chamada “Hipótese da Higiene”, formulada após um estudo conduzido em crianças desde o nascimento até os 23 anos de idade, onde foi demonstrado que crianças provenientes de famílias maiores e com irmãos mais velhos estavam significativamente mais protegidas de doenças alérgicas. Posteriormente, pesquisadores propuseram algumas hipóteses imunológicas que explicavam o fenômeno. Durante a gravidez há um desvio do sistema imune para o perfil Th2 que é refletido no recém-nascido (WARNER, 2004), os estímulos ambientais como exposições a bactérias e vírus geram um repertório de resposta Th1, um processo caracterizado por mudanças no padrão de citocinas secretadas pelas células T. Entretanto, se os estímulos ambientais forem reduzidos, e houver uma predisposição genética, um balanço disfuncional Th2 irá persistir e predispor a desordens atópicas (HOLT e JAMES, 2000).

As mais variadas infecções bacterianas, fúngicas e virais vem sendo estudadas com alguns resultados bastante controversos (MATRICARDI et al., 2000; JANSON, 2007 ; CHEN et al., 2008). Entretanto, foram as infecções helmínticas que trouxeram os resultados mais intrigantes. Sendo infecções caracterizadas por desencadearem uma resposta imune do tipo Th2, semelhante à resposta alérgica, acreditava-se que ela seria uma potencializadora das reações alérgicas. Entretanto, os achados obtidos através das pesquisas epidemiológicas e experimentais mostraram que estas infecções protegiam o indivíduo do desenvolvimento de alergias (COOPER et al, 2003; McCONCHIE et al, 2006).

Atualmente, a alergia é definida como uma doença dependente de uma resposta do sistema imune a um antígeno exógeno, sob outros aspectos, inócuo. Segundo a classificação de Gell e Coombs (1963) a resposta alérgica é ocasionada por uma reação de hipersensibilidade do tipo I ou imediato, mediada por anticorpos da classe IgE que ligam-se aos receptores de alta afinidade (FcεRI) de mastócitos e basófilos, num processo denominado de sensibilização. Num segundo contato com o alérgeno, estes se ligam a duas IgEs específicas presentes na membrana dos mastócitos levando a degranulação destas células. Os grânulos liberados são ricos em leucotrienos, histamina e citocinas pro-inflamatórias, os quais acarretam espasmo da musculatura lisa e iniciam a resposta inflamatória das vias aéreas, ocasionando coriza, espirros e broncoespasmo. Esta resposta é também responsável pela reação de hipersensibilidade imediata dos testes cutâneos aos aeroalérgenos (JANEWAY et al., 2007).

A IgE específica para estes antígenos liga-se ainda a receptores de baixa afinidade (FcεRII) de eosinófilos, linfócitos, plaquetas e macrófagos, intensificando e modulando a resposta inflamatória através da produção de IL-4, que estimula a produção de IL-5, IL-13 e demais

citocinas e moléculas inflamatórias envolvidas na resposta Th2 (BUSSE et al., 2001; MURPHY e REINER, 2002; ABBAS e LICHTMAN, 2005). Na inflamação eosinofílica das vias aéreas, a IL-5 está envolvida na diferenciação, ativação e sobrevivência dos eosinófilos, aumentando sua responsividade para a eotaxina, através da regulação da expressão de receptores CCR3 de eosinófilos para esta citocina (LEFORT et al., 1998).

A importância da IgE no curso das doenças alérgicas tem sido amplamente relatada, e estudos de bloqueio da IgE circulante com anticorpos monoclonais diminuíram não só a IgE sérica como reduziram a expressão dos receptores de alta afinidade para IgE (FcεRI), atenuando tanto a fase inicial (mediada por mastócitos) como a fase tardia (inflamatória) da asma (KON et al, 1998).

A causa da alergia é multifatorial e depende da interação de fatores genéticos, tempo e quantidade de exposição aos alérgenos, principalmente os derivados de ácaros da poeira doméstica, de animais de estimação e fungos e de fatores ambientais ainda não bem caracterizados. Os ácaros da poeira doméstica, *Dermatophagoides farinae*, *D. pteronyssinus* e *Blomia topicalis*, são os principais agentes desencadeadores de fenômenos alérgicos descritos em todo o mundo (PLATTS-MILLS et al., 2000).

Algumas propriedades do alérgeno são definitivas para sua maior capacidade de sensibilizar e desencadear a resposta imune alérgica (ABBAS e LICHTMAN, 2005). A atividade enzimática é uma das propriedades mais importantes no desencadeamento das reações alérgicas provocadas pelos alérgenos, possivelmente por destruir as junções comunicantes das células epiteliais aumentando a permeabilidade da mucosa bronquial a macromoléculas (SCHULZ et al, 1999).

Dentre as alergias respiratórias, destaca-se a asma por ser uma das doenças crônicas mais comuns na infância (WONG et al, 2001), atribuída a ativação e produção de citocinas pelos linfócitos T $CD4^+$ de forma imprópria, induzida principalmente por aeroalérgenos, resultando em inflamação eosinofílica das vias aéreas, aumento da IgE sérica, degranulação dos mastócitos submucosos do trato respiratório, constrição brônquica e secreção aumentada de muco (ANDERSON, 2002).

Atualmente, a asma é classificada em duas formas muito específicas: asma atópica e asma não-atópica. A asma atópica acomete principalmente indivíduos entre 4 e 40 anos, porém tem sido também relatada em populações geriátricas (APTER et al., 1988). Na asma existe uma relação temporal entre sintomas respiratórios e exposição aos alérgenos e presença de anticorpos IgE contra alérgenos comuns. Por outro lado, a asma não atópica acomete principalmente crianças com menos de 04 anos ou idosos com mais de 60 anos de idade. Nesta enfermidade a inflamação das vias aéreas mediada por IgE específica para alérgenos está ausente.

Embora recentes revisões tenham comentado o paralelo entre o aumento de doenças alérgicas e auto-imunes no mundo ocidental, não é fácil explicar como as mesmas mudanças ambientais podem promover o aumento de doenças com orientações imunes opostas e mutuamente excludentes (BLACK, 2001; WILLS-KARP et al, 2001). Tem sido argumentado que a secreção de citocinas anti-inflamatórias como a IL-10 produzidas pelas células T regulatórias podem ser a chave para regular ambos os desvios imunes (YAZDANBAKHSH et al, 2001). Portanto as infecções na infância ou mesmo durante a gestação exercem um importante papel protetor contra o desenvolvimento de alergia como uma conseqüência do

estímulo crônico dos receptores semelhantes ao Toll (TLRs), sendo, portanto, a causa do aumento dos fenômenos alérgicos a diminuição da atividade das células T regulatórias mais do que a exacerbação da resposta Th2 (YAZDANBAKHSH et al, 2002; ROMAGNANI, 2004).

1.3. *Toxocara canis* e alergia

Diversos estudos epidemiológicos vêm demonstrando que a infecção por *T. canis* contribui para o desenvolvimento de manifestações alérgicas no homem. Buijs et al (1997) mostrou associação entre a soropositividade para *T. canis* e asma alérgica/bronquite recorrente e aumento de IgE específica contra aeroalérgenos. Alteração na atividade respiratória e asma tem sido observada em diversos estudos em associação com a infecção por *T. canis* (FERREIRA et al, 2007; ESPINOZA *et. al*., 2008; MARTIN *et. al.,* 2008; FERNANDO et al, 2007), assim como eosinofilia (TEIXEIRA et. al, 2006; ESPINOZA *et. al*., 2008; MARTIN *et. al.,* 2008). Chiodo *et. al*., (2006) observaram que a eosinofilia estava presente em 86,95% dos indivíduos positivos para *T. canis* ($p < 0,001$, OR = 11,03) e Fernando *et. al.* (2007), no Sri Lanka, observaram que a presença de eosinofilia foi significantemente maior nas crianças soropositivas (77%) que nas soronegativas (40%) ($p<0,001$).

Paralelamente a estes achados, outros autores têm relatado ausência de associação entre soropositividade para *T. canis* e atopia e asma. Sharghi et al (2001) não observaram associação entre asma e infecção por *T. canis* em 324 crianças nos Estados Unidos, enquanto Yong-Hun et al (2008) avaliaram a associação entre eosinofilia acima de 10% e soropositividade para *T. canis* em 96 amostras de soro, mostrando ausência de correlação entre a infecção e eosinofilia.

Na tentativa de entender os mecanismos envolvidos na imunopatologia desta infecção bem como seus efeitos em patologias imunomediadas como a asma, modelos murinos têm sido amplamente utilizados com este propósito (ESPINOZA et al, 2002a; PECINALI et al, 2005; PINELLI et al 2007). Estes estudos vêm mostrando que infecção por *T. canis* resulta em inflamação pulmonar persistente, eosinofilia, aumento da produção de IgE, hiperreatividade das vias aéreas e produção de citocinas do tipo Th2 (PINELI et al, 2007).

Pineli et al (2007) estudaram os efeitos da infecção com *T. canis* nas manifestações alérgicas combinando um modelo murino para toxocaríase e um modelo experimental para inflamação das vias aéreas utilizando ovoalbumina (OVA). O efeito do tempo de infecção na inflamação das vias aéreas também foi avaliado e classificado em inicial com 3 dias e tardio com 20 dias de infecção. Foi demonstrado o aumento da expressão da citocina IL-4 e eosinófilos nos pulmões dos camundongos, sendo estes mais significativos no início da infecção. Além disso, foi observado aumento da IgE no plasma e no lavado broncoalveolar (BAL) durante a infecção inicial e na tardia.

Além da típica indução de perfil Th2 ocasionada pelo *T. canis*, moléculas relacionadas à ativação de padrão de resposta Th1, tais como o óxido nítrico, vêm sendo descrita na infecção por *T. canis* e associada a efeitos deletérios no sistema vascular pulmonar e dano direto aos hospedeiros murinos (ESPINOZA et al, 2002a). A via de sinalização citoplasmática envolvida na produção de óxido nítrico após o estímulo com antígeno de *T. canis* foi estudada em macrófagos alveolares de ratos, mostrando que a fosfolipase C e A2 induz a produção e liberação do óxido nítrico por duas vias distintas: tanto pela indução do aumento do cálcio intracelular; como pela liberação do ácido araquidônico (ESPINOZA et al, 2002b).

A lesão tecidual causada pela migração das larvas do *T. canis* pelos diversos órgãos induz um aumento de citocinas pró-inflamatórias que pode comprometer as funções sistêmicas. Pecinali et al (2005) avaliaram o nível das citocinas IL-6, IFN-γ, eotaxina e RANTES (Regulated on Activation Normal T Cell Expressed and Secreted) no plasma e no lavado bronco-alveolar (BAL) de camundongos infectados com *T.canis*. A IL-6 é um marcador bem caracterizado de resposta inflamatória bem como o IFN-γ, enquanto o RANTES é importante na resposta eosinofílica e a eotaxina é uma quimiotaxina de eosinófilos. Neste estudo todas as citocinas estavam aumentadas durante a infecção pelo parasito mostrando uma atividade inflamatória nas vias aéreas.

Diante do exposto, os achados da literatura ainda são muito controversos no que diz respeito a capacidade do *T. canis* em induzir ou proteger de alergias o que sinaliza para a necessidade de estudos mais aprofundados com este propósito. Essa disparidade entre os dados na literatura pode ser uma conseqüência do baixo número de amostras em alguns trabalhos epidemiológicos. Somam-se a isto as diferenças no diagnóstico sorológico da toxocaríase, assim como a determinação da definição de atopia e asma utilizadas pelos diferentes autores. A alta prevalência de asma e atopia em nossa população nos estimulam a buscar possíveis fatores que podem estar determinando o aumento destas doenças.

2. HIPÓTESES

2.1

H0) Infecção causada por *Toxocara canis* não está associada com o nível socioeconômico e condições de higiene da população.

H1) Infecção causada por *Toxocara canis* está associada com o nível socioeconômico e condições de higiene da população.

2.2.

H0) A presença da infecção por *Toxocara canis* não modula o sistema imune, portanto não influencia o desenvolvimento de atopia e asma

H1) A presença da infecção por *Toxocara canis* modula o sistema imune regulando o desenvolvimento de atopia e asma.

3. OBJETIVO GERAL

Estudar os fatores de risco para infecção por *Toxocara canis* e investigar a associação desta infecção com eosinofilia, IgE total, atopia e asma em crianças oriundas de população de baixa renda de Salvador, Bahia.

3.1. Objetivos específicos

- Determinar os fatores de risco para a infecção por *T.canis* nas crianças do estudo.
- Investigar possível associação entre a soropositividade para *T. canis* com eosinofilia e níveis de IgE total sérica.
- Investigar possível associação entre a soropositividade para *T. canis* com atopia e asma nas crianças do estudo.

4. RESULTADOS

4.1 *Manuscrito 1*: Risk factors for *Toxocara canis* infection in children from a Brazilian urban setting (formatado para submissão ao Transaction of the Society of Medicine and Tropical Higiene)

4.2. *Manuscrito 2:* Toxocara canis infection as risk factor for atopy and atopic asthma in a large set of children from a Latin American urban center (a ser submetido ao JACI – Journal of Allergy and Clinical Immunology)

Risk factors for *Toxocara canis* infection in children from a Brazilian urban setting

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SUMMARY

Background: Visceral larva migrans syndrome is a zoonosis caused by migration of *Toxocara sp* larvae in human organs. The improvement of its diagnosis showed that this disease occurs worldwide. This study aimed to estimate the seroprevalence of *T. canis* infection, and to identify potential risk factors for this infection in children living in poor areas of Salvador, Brazil. **Methodology:** Parents of 1,445 children answered a validated questionnaire containing possible risk factor for acquisition of this infection. Blood was collected and the presence of IgG anti-*T. canis* antibodies was detected by indirect ELISA using *T. canis* larval excretory-secretory antigens (TcESLA) in pre-absorbed sera with *Ascaris lumbricoides* extract. **Results:** Seroprevalence of *T. canis* infection was 48.4%. Among the risk factors studied, contact with dogs and cats, child´s age, low maternal scholarity and household located in paved streets were shown to be risk factors for *T. canis* infection. **Conclusions:** The seroprevalence of *T. canis* infection is high among children living in a poor urban center of Brazil. The association of the infection with cat's contact is suggestive that *T.canis* ESLA reacts with anti-*T. cati* antibodies. The finding of association of *T. canis* infection with living in paved streets may be secondary to the spreading of the eggs and higher exposure of the population to cat and dog contaminated feces in this type of ground . The relationship of low maternal education with higher infection by *T. canis* supports previous studies showing that low socioeconomic status is a risk factor for the acquisition of this infection.

Keywords: Toxocara canis, seroprevalence, children, risk factors, dog, cats, cross-reaction

INTRODUCTION

Visceral larva migrans (VLM) is a syndrome of human beings, transmitted by accidental ingestion of embryonated eggs of *Toxocara canis* (dog round worms) or rarely of *T. cati* (cat round worm). Their larvae do not migrate to intestine as occur in the definite hosts and remain migrating through the organs and visceras leading to polymorphic clinical pictures which vary from asymptomatic to severe systemic forms such as prolonged fever with hepato-esplenomegaly, meningoencephalitis and asthma-like symptoms (DEPOMMIER, 2003[1]; HARALAMBIDOU *et. al*., 2005[2]; SAPORITO *et. al*., 2008[3]). Further than the clinical pictures mentioned above, *T. canis* infection leads to a hypersensitivity reaction status, even in asymptomatic subject, which may cause eosinophilia, increase in total IgE and high susceptibility to asthma (BUIJS et. al., 1997[4]; FERREIRA et. al., 2007[5]). Although this infection occur worldwide, its prevalence is higher in non-affluent population and countries (COELHO *et. al*., 2004[6]; ESPINOZA *et. al*., 2008[7]), where its diagnosis is rarely done, being considered a neglected disease. For example in Brazil, from our knowledge, only one laboratory is able to diagnosis VLM. The disease diagnosis depended on the larvae cultivation to produce the antigen used in ELISA, but actually commercial kits are available, although expensive. This infection is also prevalent in many developed countries and its global importance may be underestimated. In the United States, it is the most common helminthic infection, affecting millions of individuals (HOTEZ E WILKINS, 2009[8]).

Strain dogs and cats and domiciliated pets from low income population play an important role in the transmition of *Toxocara* s*p* eggs providing environmental contamination, the perpetuation of the cycle and spreading the diseases among the human population. The contact with contaminated ground, hands or food with embryonated eggs is the most

commom *Toxocara sp* transmission way, but contact with these animals, and the presence of eggs in their furs has also been related to this zoonosis (WOLFE AND WRIGHT, 2003[9]). Studies in Brazil (ALCANTARA-NEVES et al.,1989[10]; ALMEIDA et. al., 2007[11]; TIYO et. al., 2008[12]) and worldwide (MIZGAJSKA, 1997[13]; DEVERA et. al., 2008[14], MARTIN and DEMONTE, 2008 [15]) show that the soil of public areas such as plazas, parks, campsites and beaches are important foci of transmission and represents an important risk factor to the human being. In addition, factors such as age, maternal education, low socioeconomic conditions, have also been related to this zoonosis (WOLFE and WRIGHT, 2003[9]). Most of these works however were carried out in small sample population of limited areas. In this study, we aimed at determining the seroprevalence of *T. canis* infection in a large set of children living in different poor areas of Salvador, a city of 2.800.000 inhabitants in Bahia and investigate the risk factors involved in its transmission, helping to understand the epidemiology of LMV in this city and similar settings around the World.

MATHERIAL AND METHODS

Study population

This study was conducted in the city of Salvador with nearly 2.800.000 inhabitants, mostly of mixed African descent. Briefly, we studied 1,445 children born between 1994 and 2001 and enrolled in a cohort recruited from 1997 and 2003 for evaluating the impact of a sanitation program on the incidence of childhood diarrhea and recruited from geographical microareas (Figure 1), selected to represent the population without sewage at that time (STRINA et al., 2003[16]). In 2005 these children were resurveyed and social, demographic and environmental data were recollected. The current work is a transversal study, which evaluated whether the *T. canis* infection status in 2005 was associated with exposures to potencial risk factors for

acquisition of the infection. Informed consent was obtained from the children's parents or guardians. Ethical approval was granted by the Instituto de Saúde Coletiva at Universidade Federal da Bahia and the National Commission on Ethics in Research (CONEP), Brazil.

Blood collection

Blood collection was carried out in laboratory facilities established in each studied area. A blood sample of 5mL was collected from each child and the sera were cryopreserved at -20 ºC until use.

Obtaining excretory-secretory larval antigen (TcESLA)

Excretory/secretory antigen of second stage larvae (L2) of *Toxocara canis* (TcESLA) were obtained following the de Savigny (1975) [17] technique, modified by Alcantara-Neves et. al. (2008)[18]. The larvae obtained were cultured in RPMI containing gentamicin (160 μg/ml) and amphotericin B (2.5 μg/ml), maintained in a 5% CO_2 chamber, at 37 °C. The culture supernatant containing the TcESLA was collected each seven days and stored at -70 °C until use. Phenylmethyl-sulfonyl fluoride (PMSF) 0.1 M was added to the collected supernatant. All reagents for the larvae cultivation and the PMSF were from SIGMA (Sigma Chemical Co., San Louis, MO,USA). The culture supernatant was concentrated in an Amicon ultrafiltration device (Millipore Corporate, MA, USA) with a cellulose filter with pore size of 3000 kD (Millipore Corporate, MA, USA), at 4° C, Fifteen ml of the concentrated supernatant were dialyzed against buffered phospate saline solution, pH 7.4 (PBS), containing 0.1 M PMSF and 0.1% of sodium azide (Sigma Chemical Co., San Louis, MO,USA). After centrifugation the supernatant containing theTcESLA was aliquoted and cryopreserved at -70 ºC until use.

Characterization of the TcESLA by sodium duodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

TcESLA SDS-PAGE was performed according to Laemmli (1970)[19] using a Mini-PROTEAN III Electrophoresis Cells (Bio-Rad Laboratories, Hercules, CA) and a 12% polyacrylamide gel in the presence of 10% sodium dodecyl sulphate (Merck & Co., Inc., White house Station, NJ, USA). Protein fractions were labeled with Coomassie Brilliant Blue R 250 (Sigma Chemical Co., San Louis, MO, USA). The relative molecular weights were calculated using prestained protein of standard molecular weight according to the relative electrophoretical mobility (REM), using the following equation: REM = distance of the protein migration/distance of bromophenol blue migration.

Sera absorption with Ascaris lumbricoides antigens

In order to eliminate cross-reaction between anti-*A. lumbricoides* and anti-*T.canis* IgG antibodies the sera were pre-absorbed with an extract of adult *A. lumbricoides* in the presence of polietilenoglicol (3%) (PEG 15.000 – Sigma Chemical Co., San Louis, MO,USA) and 0.1% sodium azide diluted in PBS. After incubation for 30 minutes in a homogenizer at room temperature, the material was centrifuged at 5,724g for 10 minutes. The supernatant containing the serum was re-absorbed as described above and frozen at -20 ºC until testing.

Immunoassay for detection of anti-Toxocara canis IgG antibodies

IgG antibodies were detected in sera by indirect ELISA assay using TcESLA as antigen. Briefly, 96-well plates were sensitized with 3.2 μg/mL of TcESLA in carbonate/bicarbonate buffer, overnight at 4°C. The plates were blocked with a solution of PBS containing 10% of fetal calf serum (FCS - Cutilab, Campinas/SP, Brazil). The sera were applayed to the wells diluted at 1:1,000 in a solution of PBS containing 0.05% tween 20 and 2.5% fetal calf serum

(PBS/T/FCS). The reaction was developed using an anti-human biotinylated IgG conjugate (BD Pharmingen, San Diego, CA, USA) diluted at 1:2000 in PBS/T/FCS followed by Streptavidin-peroxidase (Streptoavidin-HRP, BD Pharmingen, San Diego, CA, USA) diluted at 1:1000 in PBS/T/FCS. Hydrogen peroxide and OPD (o-Phenylenediamine - Sigma Chemical Co., San Louis, MO, USA*)* were used as substrate and chromogen. The plates were incubated for one hour at room temperature after each step, except for the substrate which was incubated for 1/2 hour and washings were done four times betwwen steps with PBS/T. The reaction was stopped within 30 minutes with sulfuric acid 2N and the optical density was determined using a 490nm filter. To determine the cutoff, 19 serum samples from individuals who had no contact with dog and/or cat and higher socioeconomic status were using like negative controls. The mean of optical density plus three standard deviation was considered the cutoff. To determine the avidity of the antibodies binding to the antigen, the assay was performed in duplicate, and for each serum two wells were washed after the serum incubation for 5 minutes with PBS-T containing 6M urea (Ureia P.A. – VETEC, São Paulo, Brazil). Following, the assay was continued as described above. *Toxocara*-specific IgG avidity was calculated by using the for relative avidity index: Percentage of Reduction (PR) = 100 – O.D. with urea x 100/ O.D. without urea. A serum was considered to have low avidity, when the PR was above 50% (Dziemian *et. al*., 2008[20]).

Statistical Analysis

Only children for whom complete data was available were included in the analysis. The children´s gender and age in 2005 were treated as *a priori* confounders. The following variables were studied as risk factors for acquisition of *T. canis* infection (outcome): whether the child attended nursery, maternal schooling, presence of dogs or cats at home, if house were served by a paved road. We first performed a univariate analysis between each potential risk factor and outcome; built a multivariable model with standard logistic regression

including only significant variables from the univariate analysis; and then, we assessed each non-significant variable a second time by including each one in the model (one at a time). If the variable remained non-significant, it was completely removed from the analysis. This process was repeated until no variables remained to be assessed. The association between outcome and risk factors was estimated with odds ratio and 95% confidence interval.

RESULTS

Among the original 1,445 children enrolled in the 2005 survey, 1,308 had complete data sets and were used in the analysis. No statistically significant differences were found in the frequencies of the analyzed variables, between the excluded and the studied children.

Figure 2A shows the quality of the TcESLA, used as antigen to detect anti-*T.canis* IgG in an indirect ELISA, determined by SDS-PAGE showing bands of 104, 95, 86, 71, 40, 21, 17, 16 and 13kDa. Figure 2B shows the result of the sera absortion with somatic antigen of *A, lumbricoides*. Sera absorbed twice with PEG 15,000 had a decrease of 76.39% in optical density. Figure 1C shows the determination of the assay cut-off which was performed with sera from 19 children without history of contact with dogs, and five children with history of contact with dogs and low social and economic condictions. The cut-off obtained, of 0.23 was calculated by the mean of OD of the negative controls plus three standard deviations of this mean. Figure 1D shows the results of the determination of anti-*T. canis* IgG antibodies in the whole population by indirect ELISA.

The majority of the children aged between six and seven years old (40.5%), approximately half of them were male 702 (53.6%), 214 (16.3%) had attended nursery school for some time and 64.6% of children lived in areas without paved street. 388 (29.6%) of their mothers had

completed a high school degree. Using the established cut-off, 48.4 % of the children were seropositive for *T. canis* IgG. Only 2.8% of the 633 children seropositive showed IgG of low avidity, indicating a recent infection (Table 1).

The following variables were significantly associated with an increased prevalence of *T. canis* infection at both univariated and multivariated analyses: to be ≥ 8 years old (crude OR=1.38; 95%C.I.=1.04;1.83 and (adjusted OR=1.43; 95%C.I.=1.07;1.92); living in house placed in paved streets (crude OR=1.30, 95%C.I.=1.04;1.63 and adjusted OR=1.26, 95%C.I.=1.00;1.60); presence of a dog at home (crude OR=1.45, 95%C.I.=1.16;1.81; adjusted OR=1.36; 95%C.I.=1.07;1.72) and presence of a cat at home (crude OR=1.62, 95%C.I.=1.21;2.16; adjusted OR=1.40, 95%C.I.=1.03;1.91). Day care attendance was associated only at univariated analysis (crude OR=1.42; 95%C.I.=1.05;1.90**)** and mother with both, incomplete 2nd grade and complete 2nd grade or more were negatively associated with *T. canis* infection when compared with mothers with 1st grade or less (crude OR=0.69; 95%C.I.=0.52;0.91; adjusted OR=0.72; 95%C.I.=0.54;0.96 and crude OR=0.35; 95%C.I.=0.25;0.4; adjusted OR=0.36, 95%C.I.=0.26;0.50) respectively, (Table 2).

DISCUSSION

The diagnosis of visceral larva migrans is performed almost exclusively by antibodies anti-*T.canis* due to the difficulty of parasitological and clinically diagnosing the disease. Many laboratories have developed *in house* assays for research purpose in order to determine the prevalence of this zoonosis (AGUIAR-SANTOS, 2004)[21]. The TcESLA obtained in our laboratory had bands with similar molecular weight as antigens previously described by other authors (RUBINSKY-ELEFANT et. al., 2006[22]; IDDAWELA et. al., 2007[23]) and the standardization provided more specificity for our assay when compared with the literature,

since the serum dilution was 1:1000 instaed of 1:200 (NUNES et al. 1997)[24]. Roldán and Espinoza (2006)[25], because we used a biotin instead of a peroxidase-congugate. Even using the cut-off of the mean of negative controls plus 3 standard deviation we had a cut offt of 0.23 and some of the positive sera with optical density values above the up detection limit. Furthermore we absordeb the sera with *A. lumbricoides* extract instead of *A. suum* as reported by the above cited works. Absorption of sera with antigens from other parasites is a practice that increases the specificity of the test, since many parasite species share similar antigens giving rise to cross-reactivity between these antibodies (ISHIDA et al., 2003)[26]. For the diagnosis of toxocariasis is usually the serum samples are absorbed with *Ascaris suum* antigen (LYNCH et. al., 1988[27], NUNES et al. 1997[24]; CAMPOS JUNIOR et. al. 2003[28]; ROLDÁN et al. 2006[25]). Nunes et al. (1997) [24] determined at least one band of molecular weight around 55-66 kDa responsible for cross-reactivity between *T. canis* and *A. suum*, and that disappeared with the pre-absorption of sera with antigen *A. suum*. Roldán and Espinoza (2009) [29] determined antigenic bands recognized by sera from patients with toxocariasis as following, 24, 28, 30, 35, 56, 117, 136 and 152 kDa. However, only the bands of 24-35 kDa were highly specific for *Toxocara* infection (98.3%), while the other was observed cross-reactivity with sera from patients infected with other helminthic infections. In our population the helminth infection are caused only by *A. lumbricoides* and *Trichuris trichiura* which occur in 16.1% and 10.8% respectively in our children. The absorption with antigen *Ascaris lumbricoides* decreased up to76.39% of optical density, indicating a higher removal of specific antibodies to *A. lumbricoides* avoiding cross-reactions between this helminth and anti-*T. canis* in antibodies. Additionally we performed absorption with *T. trichiura* somatic antigen after absorption with *A. lumbricoides* antigen and observed not significant decrease in optical density that justify its use, showing that pre-absortion with *A. lumbricoides* was sufficient to avoiding cross-reaction with others helminthes (data not shown).We also have

also absorbed the sera with *Ancylostoma braziliensis* antigens and there was no decrease of the anti-*T. canis* IgG, showing that this parasite do not share antigens reactive with IgG (data not shown)..

In our work we found a prevalence of IgG anti-*T.canis* of 48.4%. Others studies conducted in Latin America and Brasil reported small prevalences, except for Damian et. al. (2007)[30] who found a prevalence of 52% among adult population in Amazonas state in Brazil. Alonso et. al. (2000)[31] reported a positivity of 37.9% in children younger than 14 years in Argentina, Espinoza et. al. (2008)[7] determined a seroprevalence of 32.4% in Peru and in Brazil, Chieffi (2009)[32] in a review, cited prevalence of *T. canis* varying from 3.72% to 40%. In agreement with our results, Radman et. al (2000) [33] in Argentina observed a prevalence of 39% infection.

Maternal education is an indicator of socioeconomic status of the family. The result of the seroprevalence of IgG anti-*T.canis* found in this study was similar to those observed in other low-income populations, where prevalence of infection in children of mothers with fewer years of education was higher. Alderete et. al. (2003)[34] diagnosed a prevalence of 38.8% in children with a mean age of 9.4 years and determined that *Toxocara* infection was inversely proportional to family income.

Contact with dogs has been shown in several studies as an important risk factor for toxocariasis. A cross-sectional study estimated a frequency of 52% positivity for *T.canis* in 34 families in the Amazonas state. Individuals who had contact with dog at home, 60% were positive (chi2 = 14.317, p = 0.026), and who had contact with puppies at home, 66.6% were positive (chi2 = 22.149, p = 0.008), demonstrating the association between contact with the dog and the presence of anti-*T.canis* IgG (Damian et. al., 2007)[30]. In Argentina, Chiodo et. al. (2006) [35] evaluated 100 individuals for IgG anti-*T. canis* and 23% were positive, and all had

contact with dog at home. Our results confirm these findings of the presence of the dog at home as a risk factor for *T.canis* infection in this study population.

Several epidemiological studies indicate contamination of soil as a determinant in infection by *T. canis*. In the present study it was noted that paved street increased the chance of infection, which makes us suppose that maybe dog and cat may defecate in those streets and the absence of soil to absord the eggs may favour more contact of the children with contaminated feces. Cross-reaction of IgG between the *T. canis* and *T. cati* ESLA occur (KENNEDY et al, 1987)[36]. Few studies were conducted to estimate the infection of cats and their potential role as reservoir for human toxocariasis, Martinez-Barbosa et. al. (2003)[37] determined a prevalence of 42.5% of *T. cati* infection in cats which makes one think that this parasite may be common and raises the importance of the development of a species-specific ELISA for detection anti-*Toxocara sp* IgG, useful for studies on the epidemiology of LMV caused by both *Toxocara* species.

Some studies refer that soil contamination is not the only effective route in human toxocariasis and eggs of *T. canis* can be sprouted in fur and direct contact between humans and dogs may be an alternative route of human infection (WOLFE and WRIGHT, 2003) [9]. Aydenizoz-Ozkayhan et. al. (2008) [38] collected 51 fur samples and observed that 21.56% of the dogs had eggs in their fur. Roddie et.al. (2008) [39] examined 100 dogs for the presence of eggs in fur and found Toxocara eggs in 67% of adult dogs and 95% of puppies. In the Netherlands, Overgaauw et al (2009)[40] found *Toxocara* eggs in 4.4% of dog fecal samples and in 12.2% of their fur samples. Moreover, many of the owners allowed their dogs to climb and sleep in their beds, and only 15% washed their hands after contact with your pet. Therefore, this close physical contact between pets and their owners possibly increase the risk of

transmission of *Toxocara sp* and point to the need for greater attention to the potential risk to which humans are exposed.

In conclusion this work shows that *T. canis* is a highly prevalent infection in the studied population and it is closely related to social status and hygiene. The presence of the dog at home proved to be an important risk factor for this disease and is necessary to adopt sanitary measures more specific for resident dogs, since only control programs stray dogs is not the only way to control the disease. The association with presence of cats in house is suggestive that thre are antigenic similarities between *T. canis* and *T. cati* ESLA and that anti-*T. cati* antibodies have influenced the outcome of the study. Paving the street which was associated with increased risk of *Toxocara* infection may be influencing the increased exposure to dog and cat feces probabily because the absence of soil to absorb the eggs make them to be more exposed and accessible to be transmitted to the children.

Statements on the authors' contributions

LRM has done the laboratory assays and wrote the first draft of the manuscript. VCCD helped in the laboratory assay and in analyze the data. CAF, has helped in obtaining the *Toxocara* antigen and revised the manuscript. RE and RLF have analysed the data: ; LPC has helped in the *T. canis* assay standardization and revised the manuscript. MLB has coordinated the epidemiological work. NMAN planned the work, supervised the laboratory work, helped in the manuscript elaboration and revised the text.

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Ethical approval

Ethical approval was obtained from the Brazilian National Ethical Committee. Written, informed consent detailing all procedures to be carried out on the children was signed by the parents or legal guardian of each child.

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Legend to Figures:

Figure 1. Aerophoto of the city of Salvador. The red spot are the microaraes chosen to represent the city areas without sewage system.

Figure 2. Standardization procedures for immunoassay to detect anti-T. canis IgG and results of the tested sera. **A**. 12% polyacrilamide gel eletroforesis of TcESLAof with blue Cooumasie (a) molecular weight markers; (b, c and d , antigen dilutions of 10, 20, 40 and 80µg/ml respectively); **B**. Results of the absortion of the children sera with somatic antigen of adult *A. lumbricoides*.in the presence of poliethileneglicol as described im Material and Methods: C. Determination of the ELISA for anti-*T.canis* IgG cut-off as described in Material and Methods and E. Dispersion of the anti-*T.canis* IgG in serum samples of the study population.

Table 1. Frequency of variables relevant to the study of risk factors for infection *T.canis* collected from the ISAAC phase II questionnaire**.**

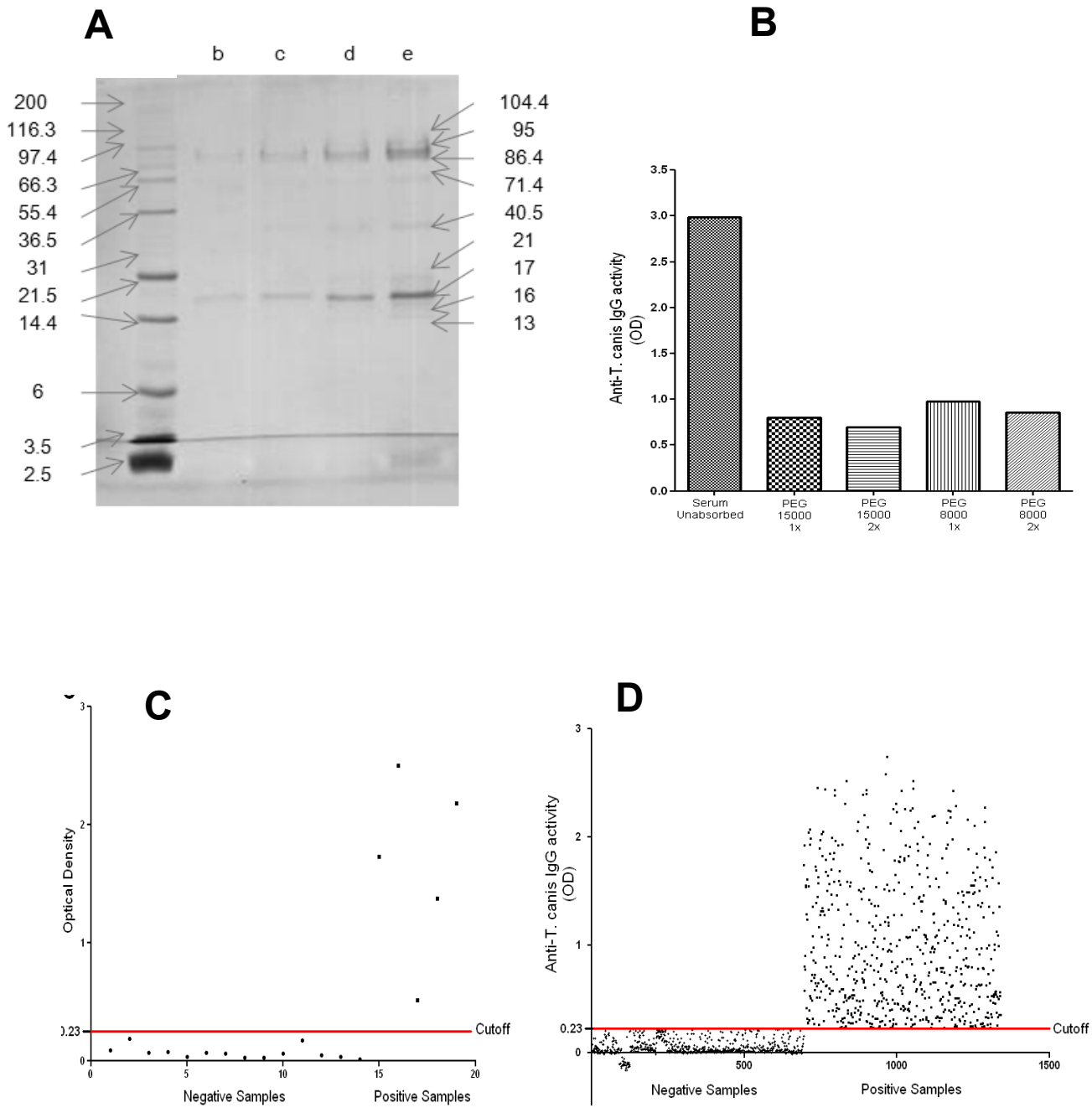
Variables	**N**	**%**
Age		
≤ 5 years	336	25.7
6-7 years	530	40.5
≥ 8 years	443	33.8
Gender		
Male	702	53,6
Day care attendance		
Yes	214	16.3
Maternal scholarity		
1st grade or less	293	22.4
Incomplete 2nd grade	628	48.0
Complete 2nd grade or more	388	29.6
Street paviment		
Yes	464	35.4
Dog at home		
Yes	517	39.5
Cat at home		
Yes	232	17.7
Anti-*T. canis* IgG		
Positive	633	48.4
Anti-*T canis* IgG of low avidity	18	2.8

Table 2. Association between seropositivity for *T.canis* IgG and the studied risk factors for acquisition of this infection in 1309 children.

Variables	Anti-*T.canis* IgG positivity		
	n(%)	Crude OR (95% C.I.)	Adjusted* OR (95% C.I.)
Gender			
Female	289 (47.6%)	1	1
Male	344 (49.0%)	1.06 (0.85; 1.31)	1.03 (0.82; 1.29)
Age			
≤ 5 years	152 (45.2%)	1	1
6-7 years	245 (46.2%)	1.04 (0.79; 1.37)	1.04 (0.79; 1.38)
≥ 8 years	236 (53.3%)	**1.38 (1.04; 1.83)**	**1.43 (1.07; 1.92)**
Maternal scholarity			
1st grade or less	177 (60.1%)	1	1
Incomplete 2nd grade	322 (51.3%)	**0.69 (0.52; 0.91)**	**0.72 (0.54; 0.96)**
Complete 2nd. grade or more	134 (34.5%)	**0.35 (0.25; 0.47)**	**0.36 (0.26; 0.50)**
Day care attendance			
No	514 (46.9%)	1	1
Yes	119 (55.6%)	**1.42 (1.05; 1.90)**	1.27 (0.94 ; 1.73)
Street paviment			
No	389 (46.0%)	1	1
Yes	244 (52.6%)	**1.30 (1.04; 1.63)**	**1.26 (1.00; 1.60)**
Dog at home			
No	354 (44.7%)	1	1
Yes	279 (54.0%)	**1.45 (1.16; 1.81)**	**1.36 (1.07; 1.72)**
Cat at home			
No	498 (46.2%)	**1**	1
Yes	135 (58.2%)	**1.62 (1.21; 2.16)**	**1.40 (1.03; 1.91)**

Bold numbers are statistically significant (P< 0.05)

*Adjusted by all variables


A
b
c
d
e
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
6
3.5
2.5
104.4
95
86.4
71.4
40.5
21
17
16
13
B
Anti-T. canis IgG activity (OD)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Serum Unabsorbed
PEG 15000 1x
PEG 15000 2x
PEG 8000 1x
PEG 8000 2x
C
Optical Density
3
2
1
0.23
0
Cutoff
0
5
10
15
20
Negative Samples
Positive Samples
D
Anti-T. canis IgG activity (OD)
3
2
1
0.23
0
Cutoff
500
1000
1500
Negative Samples
Positive Samples

Toxocara canis infection as risk factor for atopy and atopic asthma in a large set of children from a Latin American urban center

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Author´s contribution

Conceived and designed the experiments: MLB, LR, PJC, NMAN; Performed the laboratory work : LRM, VCCD and RVV; Contributed to development of the laboratory assays: LPC, CAV; Supervised the laboratory work: NMAN; Analyzed the data: RE, RF, RVV; Supervised the field work: MLB; LCR; Wrote the paper: LRM, CAVF, NMAN; Revised the paper: NMAN; LPC, PJC.

ABSTRACT

Background: *Toxocara canis* is a helminth of dogs which can infect human causing *Visceral Larva Migrans* (VLM) as asymptomatic infection or with unspecific clinical signs associated with eosinophilia and asthma-like symptoms. **Objectives:** To study the risk factors for acquisition of *T. canis* infection, and to investigate possible associations between this infection with specific IgE, skin prick test (SPT), asthma and IL-10 production in children. **Methods:** Parents of 1,445 children answered questionnaires, and total and specific IgE for aeroallergens and anti-*T. canis* IgG were measured. Statistical analyses estimated odds ratio (OR) and 95% confidence intervals (CI) by univariate, multivariate and polytomous logystic regression. **Findings:** The prevalence of *T. canis* infection was 47%, 22.4% of the children had asthma, specific IgE to at least one of the tested aeroallergens was positive in 48.5% (cut-off ≥0.35 kU/L) and 36.8% (cut-off ≥0.70 kU/L). Skin test reactivity for at least one of the tested allergens was present in 30.4%. *T. canis* was positively associated with eosinophilia at 4% and 10%, total IgE, specific IgE for aeroallergens but it was negatively associated with SPT and not associated with asthma. Furthemore, it was also associated with more spontaneous production of IL-10. **Conclusion:** *T. canis* infection had high prevalence in this population and it seems to be a risk factor for eosinophilia, total IgE and specific IgE but it may down modulate skin hipersensitivity and do not increase asthma in the studied children. The mechanisms for these diverging actions maybe the presence of non-funtional polyclonal IgE competing for the effector cells FCεRI with anti-allergen IgEs or/and the dowm modulation of mast cell degranulation by IL-10 stimulated by the infection.

CLINICAL IMPLICATIONS

Association between *T.canis* infection and clinical characteristics of atopy, like skin prick test positivity and increased total and specific IgE, showed in our results could improve therapeutic approaches and knowledge in atopic diseases.

CAPSULE SUMMARY

A detailed investigation of the factors involved in increasing allergy and asthma showed that *T. canis* was a risk factor to increased total IgE, specific IgE for aeroallergens, eosinophilia and IL-10 production.

KEY WORDS

Toxocara canis, eosinophilia, atopy, asthma, cytokines

INTRODUCTION

The prevalence of allergic diseases has been growing at a rapid pace throughout the World, especially in large urban centers with westernized lifestyle [1]Von Mutius et al, 1998). A better understanding of the causes and risk factors related to this growing epidemic is an important approach to prevention of these diseases [2](Franco & Pritchard, 2005). Epidemiological studies conducted in various locations around the World, have pointed out to the ability of certain helminth infections to reduce or exacerbate allergic diseases [3,4,5](Yazdanbakhsh et. al., 2001; Fallon e Mangan, 2007; Cooper, 2009).

T. canis infection has been related to changes in the immune system associated to atopy and respiratory allergies [6](Cooper, 2008). The migration of the larvae in the body gives rise to a pulmonary infection, often confused with asthma [7](Despommier, 2003) which has been shown to increases the predisposition to the development of allergic diseases especially in children [8](Chan et. al., 2001). It has been demonstrated that infection with *T. canis* increases the levels of specific IgE against aeroallergens [9](Buijs et. al.; 1997), serum total IgE, eosinophil count [9](Buijs et. al., 1997) and predispose to the development of atopic asthma in children [10](Kustimur et. al.; 2007). However, some authors have shown a negative association between seropositivity for this infection and reactivity to skin prick test (SPT).

Some authors suggest that *Toxocara* infection may contribute to the pathogenesis of allergic diseases and atopy [8](Chan et. al., 2001), however the results obtained by different research groups are contradictory [11,9,8,12,13] (Buijs et al., 1994, 1997; Chan et al., 2001, Lynch et al., 1993; Sharghi et al., 2001). Therefore, the aims of this study were to determine the association of seropositivity for *T. canis* with eosinophilia, total and aeroallergen specific IgEs, SPT, asthma (wheezing plus symptoms) and IL-10 produced by unstimulated white blood cells.

MATERIAL AND METHODS

Study population

This work was performed in Salvador, a 2,800, 000 population city of Northeastern Brazil in a cohort of 1,445 children 4 to 12 years old, living in non-affluent scattered areas, chosen to represent non-sanitated areas of the city, who had been previously investigated in 1997 to 2001, to assess the impact of a sanitation program on the occurrence of diarrhea [14Barreto ML et al, 2007]. The children were resurveyed in 2005 for studing risk fact for asthma and allergies [15 Barreto ML et al., 2006]. The legal guardian of each child filled out an ISAAC Phase II - based questionnaire. Social, demographic and environmental data were recollected using validated questionaire. Informed consent was obtained from the children parents or guardians and ethical approval was granted by the Instituto de Saúde Coletiva at Universidade Federal da Bahia and the National Commission on Ethics in Research (CONEP), Brazil.

Parasitological analysis

Paired stool samples from each child were collected two days apart and analyzed for parasites. Stools were analyzed using the gravitational sedimentation technique of Hoffman, Pons & Janner [16] to detect eggs of *Ascaris lumbricoides, Trichuris trichiura*, hookworms and *Schistosoma mansoni*. Because the last two worms were rare in the children, they were dropped from the analyses. Quantification of helminth eggs was performed using the Kato-Katz technique [17](Katz et al, 1972). All children with positive results were treated with appropriate anti-parasitic drugs [15](Barreto et al, 2006).

Collection of blood, eosinophils counting and skin prick tests

The children were referred to an ambulatory facility set up in each area of study where they were attended by a team of medical, laboratory technician and nursing students who performed the blood collection and skin testing for airborne allergens. In addition, the results of the stool examinations were given to the guardians and prescription and dispensation of medicines for helminths and protozoa were accomplished. EDTA blood samples collected from children were used for total and differencial blood cell count in automated counter (Counter Electronics Hialeah FL, USA), plasma collection for anti- *T. canis* IgG detection, and blood cell cultivation for IL-10 detection in the culture supernatants.

SPTs were applied in the right forearm of each child using extracts (ALK-Abello, São Paulo, Brazil) of *Dermatophagoides pteronyssinus, Blomia tropicalis, Blattella germanica, Periplaneta americana*, fungi, and dog and cat danders. Saline and histamine were used as negative and positive controls, respectively, following the manufacturer´s instructions. The reaction was read after 15 minutes of application of the allergens and was considered positive when the average of the two major test wheal diameters was greater than three millimeters of the two largest diameters of the wheal of the negative control.

Detection of serum anti-Toxocara canis IgG antibodies

IgG antibodies against *T. canis* were detected in sera by indirect ELISA, using as antigen, excretory/secretory products of *T. canis* larvae (TcESLA) obtained according to de Savigny and collaborators (1975)18 modified by [19 Alcantara-Neves and collaborators (2008)]. Briefly, 96-well plates were sensitized with 3.2 μg/mL of TcESLA in carbonate/bicarbonate buffer, overnight at 4°C. The plates were blocked with phospate buffered saline, pH 7.4 (PBS), containing 10% fetal calf serum (Sigma, St Louis, MO, USA). *A. lumbricoides* pre-absorbed sera diluted at 1:1000 in a solution of PBS, containing 0.05 tween 20 and 2.5% fetal

calf serum (PBS/T/FCS) were added to the plates. After incubation a biotinylated anti-human IgG (BD, Pharmigen., San Jose, CA, EUA) was added, followed by streptavidin-peroxidase (BD, Pharmigen., San Jose, CA, EUA), hydrogen peroxide and o-Phenylenediamine (Sigma, St Louis, MO, USA) used for developing the reaction. Between all steps the plates were washed for three times with PBS/T and once with PBS and all incubation were done for one hour at room temperature, except for the streptavidin-peroxidade and the substrate which were incubated for ½ hour. The reation was blocked with sulfuric acid 2N and read using a 490 nm filter spectrophotometer (Biotek EL-800, CA, USA).

Detection of total and specific IgE to aeroallergens and A. lumbricoides

The measurement of total IgE was performed as previously described [20] (Figueiredo et al, 2010). Briefly, high binding microassay plates (Costar, Cambridge, Me, USA) were coated with 4 μg/mL of an anti- human IgE antibody (Pharmingen, San Diego, CA, USA) overnight at 4^{o}C. Plates were blocked with PBS, containing 15% FCS, 0.05% tween 20 overnight at 4°C. Samples were diluted 1:10 in PBS/FCS/T and incubated overnight at 4°C. Following they were incubated in 3 steps with biotinylated anti-human IgE (Sigma Chemical Co., San Louis, MO,USA), streptavidin/peroxidase (Pharmigen., San Jose, CA, EUA) and H_2O_2/OPD substrate (Merck & Co., Inc., White house Station, NJ, USA). Washings, incubations and reading were carried out as described in the assay above. A pool of parasite infected children' sera was used as positive control. Umbilical cord serum from a newborn of a non-atopic and non-parasitized mother was used as negative control. The assay cut-off for total IgE was determined as the median plus the semi-interquartile deviation of negative controls (one hundred sera from children with 3 negative stool samples collected serially, specific IgE <0.35 and eosinophils less than 2% in peripheral blood).

Determination of specific IgE serum anti-*Blomia tropicalis, Dermatophagoides pteronyssinus, Periplaneta america, Blattella germanica* and *A. lumbricoides* concentration was carried out using the ImmunoCAP assay (Phadia Diagnostics AB, Uppsala Sweden). The IgE cut-offs of ≥≥0.35kU/L or 0.70kU/L L were considered for the analyses.

Spontaneous production of IL-10

Heparinized whole blood was cultured at a 1:4 dilution in RPMI containing 10 mM glutamine (Sigma, St. Louis, MO, USA) and 100 mg/ml gentamicin (Sigma, St. Louis, MO, USA). Pookweed mitogen (Gibco, Auckland, NZ) was used as positive control at a concentration of 2.5 mg / ml and RPMI as negative control. Cultures were incubated in humid atmosphere of 5% CO2 for 24 hours to detect IL-10. After the incubation period, the supernatants were collected and stored in a freezer-70ºC. Interleukin 10 (IL-10, BD PharMingen Duo Set Pharmigen BD, San Diego, Ca, USA) was measured in the culture supernatant using a commercial kit, employing the technique of capture ELISA following the manufacturer's recommendations.

Definition of asthma and atopy

Children were classified as having asthma in 2005 if parents reported wheezing in the previous 12 months and at least one of the following: (i) diagnosis of asthma ever; (ii) wheezing with exercise in the last 12 months; (iii) 4 episodes wheezing in the last 12 months; (iv) waking up at night because of wheezing in the last 12 months. All other children were classified as non-asthmatic.

Serum sensitivity for each child was defined by the presence of at least one detectable allergen specific IgE, and the highest level observed for any specific IgE defined a child’s

allergen specific IgE status. Atopy was defined by the presence of allergen specific IgE for at least one allergen ≥ 0.35 kU/ L or ≥ 0.70 kU/L regardless the SPT results.

Statistical Analysis

For the association between *T. canis* (exposure) and the outcomes: eosinophilia, total IgE specific IgEs and SPT, it was performed univariated and multivariated analyses, using logistic regression. Moreover, for the association between the infection and different allergy and asthma phenotypes polytomous analyses were performed. In all statistic models the following variables were considered potential confounders: gender, age, maternal schooling, parental asthma, mould, sewage system and intestinal helmith infection.

After the univariate analysis, we applied two filters. The first using a set of *a priori* variables such as gender, age, mother´s educational level, parents' asthma and the second set which included the presence of *A. lumbricoides* and *T. trichiura* infections in order to assess whether the associations obtained were being influenced by the presence of other helminth infections. In addition, it was performed polytomous analysis, which allowed us to assess in more detail how *T. canis* infection may influence the development of asthma and atopy.

RESULTS

From the 1445 children enrolled in the study, 1,148 with complete data setting were analysed. No statistically significant differences were found in the prevalence of the outcomes between the excluded and the studied children (data not shown). The demographic, social and environmental variables as well as and the infection status of the study population are presented in Table 1. 53.7% of the children were male, 40.5% aged between six and seven years, mothers' education below complete the second grade was found in 70.2% and parental asthma occurred in 13,4% of the children. Inspection of the household

by the interviewers showed that 69.9% had mold on their walls and sewage system was found in 70.1%. *A. lumbricoides* and *T. trichiura* eggs in stool were found in 14.9% and 13.8% of the children respectively. 47% of the children had serum IgG anti-*Toxocara canis* and in 3.3 % of them were low avidity, which means that the infection was in acute phase.

Table 2 presents the prevalence of the studied outcomes. Eosinophilia above 4% and 10% was found in 74.2% and 25.4% of the children respectively. 59.6% of the children had total IgE above the assay cut-off. 48.5% and 36.8% had specific IgE for at least one of the aeroallergen studied using cut off points of ≥0.35kU/L or ≥0.70kU/L, respectively. Positive SPT to at least one of the tested allergens was present in 30.4% of the children and 22.4% of the children were considered asthmatics.

Table 3 presents the association between *T. canis* infection and blood eosinophils and total IgE in the 1148 studied children. Only the results of adjusted analysis was presented. We observed a positive association between *T. canis* infection with total IgE (adjusted OR = 1.45; 95% CI = 1.12; 1.87), eosinophilia of 4% (adjusted OR = 2.89; 95% CI = 2.11;3.94) and of 10% (adjusted OR = 2.25; 95 % CI = 1.66; 3.04). Table 4 shows the association at both crude and adjusted analyses of *T. canis* infection with specific IgE (sIgE) and SPT with. adjustment was done with all counfunding variables, including intestinal helminth infections. At ≥ 0.35 kU/L sIgE cut-off, both crude and adjusted analyses were positively and significantly associated (crude OR=1.44; 95% CI =1.14; 1.82 and adjusted OR=1.48; 95%CI= 1.15;1.91). At ≥ 0.70 kU/L cut-off although both analyses showed positive association, only the adjusted analysis was statiscally significant (adjusted OR=1.36; 95%CI=1.04; 1.77). Meanwhile, negative associations with SPT were found in both analyses but only in the crude analysis there was statistical significant (crude OR = 0.73 95% CI = 0.56; 0.94). To investigate if *A. lumbricoides* and *T.trichiura* infection could modify the effect of *T. canis* infection, logistic analyses were performed in a sub-group of 914 children non-infected by

these helminths (Table 5). Only the adjusted analysis was shown. We observed that the associations with IgE cut-offs were maintained positively and statistically significant and with SPT it not only maintained negatively associated but became statistically significant (adjusted OR = 0.73; 95% CI = 0.54; 0.98).

The association of *T. canis* nfection with asthma was investigated by polytomous analysis using as the reference group non-atopic and non-asthmatic children (healthy), and to study atopic asthma a atopic and non-asthmatic group was also used.. Using the healthy group the infection was not associated with non-atopic asthma, but it was positively and statistically associated with atopic asthma adjusted OR=1.62; 95%CI=1.06;2.48). When the atopic and non-asthmatic group was used as reference this significance was lost.

Figure 1 showed that *T. canis* infection was positively and significantly associated to spontaneous production of IL-10 in whole blood culture in crude analysis (OR = 1.97 95% CI = 1.29; 3.00) and in adjusted analysis without intestinal helminth infection (adjusted OR = 1.84 95% CI = 1.20; 2.83). When the helminth were included as counfound variables it lost the statistical significance (adjusted OR* = 1.41 95% CI = 0.91; 2.17).

DISCUSSION

The syndrome of *visceral larva migrans* (VLM) is a disease of difficult diagnosis, especially because most of the infections are asymptomatic. This absence of symptoms sometimes leads to the believe that the disease has a low incidence. However, high seroprevalence has been reported in different locations in both developed and developing countries. [21]Won et. al. (2008) found a prevalence of 13.9% in the U.S., while in Latin America, [22]Alonso et. al. (2000) reported the positivity of 37.9% in children in Argentina, [23] Espinoza et. al. (2008) determined the seroprevalence of 32.4% in Peru, [24]Coelho et. al. (2004) described the

prevalence of 38.3% in children in Brazil and in Amazonas, [25]Damian et. al. (2007) found a seroprevalence of 52%, rates similar to those found in our study.

Toxocara infection has peculiar characteristics. Parasite can not complete their life cycle, remain in the larval stage in the host for long time [26](Magnaval et al, 2001). Although difficult to diagnose clinically, signs as eosinophilia, increased serum IgE, and in some cases, pulmonary disorders like-asthma can be observed in the disease course [7](Despommier, 2003). Clearly, this is immunological profile resulting from the stimulation of Th2 cells which has been also associated with atopic asthma and allergies in general. Moreover, it has been observed in mice infected with *T. canis*, increased inflammatory activity, intense eosinophil migration to the lungs and increased plasma levels of IL-6, IFN-γ, eotaxin and regulated upon activation, normal T-cell expressed and secreted chemokine (RANTES), the former two important markers of inflammatory response and the latter two of eosinophilic response [27](Pecinali et al, 2005).

Our findings suggest that *T. canis* infection is associated with eosinophilia in both cut-offs of ≥4% and ≥10%, even when adjusted for other helminth infections demonstrating the ability of *T. canis* to induce eosinophilia. A study conducted in Argentina showed that eosinophilia was diagnosed in 86.95% of the *T. canis* infected individuals. [23]Espinoza et. al. (2008) determined a frequency of seropositivity for T. canis, 32.4% and 77.96% of positive children had respiratory symptoms and 38.98% had mild to moderate eosinophilia.

The cutoff for allergen specific IgE of the Pharmacia ImmunoCAP system is standardized in ≥ 0.35 kU/L, and refers to the detectable level of IgE that this positively associated with atopy in developed countries. However, in tropical and developing countries, where there is high prevalence of intestinal parasites, this cutoff may not be ideal, since most individuals have circulating IgE [28,6](Baqueiro et al 2007; Cooper, 2008), many of them cross-reactive among parasites and allergens[29](Acevedo et al, 2009). In the present study it was used,

therefore, two cutoff for specific IgE, of ≥0.35 and of ≥0.70 kU/L the second being more representative of atopy for our study population because IgE may be increased due to high parasites burdens in tropical countries. Our analysis showed that both specific IgE against aeroallergens ≥ 0.35 as ≥0.70 proved to be positively associated with infection by *T. canis*.

Our results showed a negative association between *T. canis* infection and SPT. The absence of skin reactivity despite high IgE values has been attributed to the following mechanisms: a. high production of polyclonal IgE induced by the parasite that alters the late effector phase of Th2 allergen-specific saturating mast cells and preventing cross-linking of anti-allergen IgEs with allergens, also known as "IgE blocking hypothesis" [30,31](Holt et al, 1999; Yazdanbakhah et al, 2002) and b. the so-called "modified Th2 response." Helminth infection estimulates high IL-10 production that induces the class-switching to IgG4 which interacts with the allergens by preventing their binding to anti-allergen IgE present on mast cells and the release of this cell inflammatory mediators [32](Platts-Mills et al, 2001). And our data had shown that *T. canis* infection was associated with the increase of basal IL-10.

Moreover, *T. canis* infection was not associated with asthma in the multivariate analysis (data not showed). In the polytomous analysis *T. canis* infection was not associated with non-atopic asthma; it was associated with the phenotype of atopic non-asthmatic and apparently with atopic asthmatics (when it was compared with healthy group), but, the comparison with this phenotype, using the atopic non-asthmatic as reference, lost the association with atopic asthma. And we believe that the analysis using as reference the group of atopic non-asthmatic children would be more representative to observe the association with atopic asthma, since in many children both phenotypes coexist, atopic asthma as classified in this study could be the coexistence of atopy and asthma and not exactly asthma caused by atopy. Therefore the results of the polytomous analysis using the reference group non-atopic asthmatics showed us

that infection with *T. canis* is not associated with atopic nor non-atopic asthma and, therefore, the positive association of *T. canis* infection is exclusively to atopy.

Although it was reported in the literature pulmonary inflammatory action caused by *T. canis* infection in mice [33,34](ESPINOZA et al, 2002a, b) and asthma-like symptoms in humans, these findings were not observed in our study. It is known that *T. canis* induces different clinical patterns and that these depend on the period of infection [27](PECINALI et al, 2005). So, we believe that the timing of infection may explain this fact, since the minority of individuals in our population were positive for recent infection, demonstrated by anti-*T.canis* IgG of low avidity (3.3%). Pinelli et al (2007)[35] demonstrated that the timing affected the airways responsiviness (AHR) and the number of eosinophils in BAL of mice. While these parameters were significantly higher in early infection, production of IgE was the opposite observed, being higher only in chronic infections. Added to this the fact *T. canis* infection was positively associated with the spontaneous production of IL-10, which leads us to believe that chronic infection by *T. canis* has an immunomodulatory capacity that explains the lack of association with asthma and the down modulation of SPT in this population. The findings of association with asthma so reported in the literature [9,36 (Buijs et al, 1997; Ferreira et. al., 2007) may be due to population differences, the number of individuals of the study groups or difference in the statistical analyses.

The association between respiratory allergies and atopy in helminth infections deserve further investigation for a deep understanding of the epidemiology of asthma and atopy. A detailed investigation of the factors involved in increasing allergy and asthma in the World is important for more effective prevention and control. Studies of *T. canis* infection, until recently considered a neglected desease, has been gaining some attention for its high prevalence around the World and as an important risk factor for atopy and alteration of the

immune system. However other studies must be carried out to disantangle the role that this infection plays in the human being immune system.

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Legend to Figure 1.

Association between *T. canis* infection and spontaneous production of IL-10 in whole blood culture of 1,133 studied children. The results was showed in uni and multivarite logistic regression (odds ration and confidence interval of 95%). Crude analysis (OR = 1.97 95% CI = 1.29; 3.00); Adjusted* - analysis adjusted by potencial confounders (gender, age, maternal schooling, parental asthma, mould and sewage system) without helminth infection (OR = 1.84 95% CI = 1.20; 2.83) and Adjusted**-analysis adjusted by all potencial confounders, including helminths (OR = 1.41 95% CI = 0.91; 2.17).

Table 1: Distribution of absolute values and percentages of demographic, social and infectious variables of 1148 studied children.

Variables	N	(%)
Gender - Male	616	53.7
Age		
≤ 5 years	298	26.0
6-7 years	465	40.5
≥ 8 years	385	33.5
Maternal Scholarity		
1st grade or less	251	21.9
Incomplete 2nd grade	554	48.3
Complete 2nd grade or more	343	29.9
Parental Asthma	154	13.4
Mold at home	803	69.9
Sewage system	194	16.9
***A. lumbricoides* eggs**	171	14.9
***T. trichiura* eggs**	158	13.8
Anti-*T.canis* IgG	540	47.0
Anti-*T canis* IgG of low avidity	18	3.3

Table 2. Distribution of absolute values and percentages of the studied outcomes in children of the study

Variables (N)	Positivity	(%)
Eosinophils≥4% (1148)	852	74.2
Eosinophils ≥ 10% (1148)	292	25.4
Total IgE ≥ 0.2 **(1148)**	684	59.6
IgE ≥0.35 (1148)	557	48.5
IgE ≥ 0.70 (1148)	422	36.8
SPT≥ 3 mm **(1148)**	351	30.6
Asthma (1148)	258	22.5
IL-10 (1133)	101	8.9

Tabela 3: Association of *T. canis* infection and total IgE , eosinophilia at 4% and 10% in 1,148 studied children

***T .canis* infection**	**Total IgE**		**Eosinophilia (4%)**		**Eosinophilia (10%)**	
	n (%)	*Adjusted OR (95%C.I.)	n (%)	Adjusted OR (95%C.I.)	n (%)	Adjusted OR (95%C.I.)
Negative	330(48.2)	1	392(46.0)	1	97 (33.2)	1
Positive	354(51.8)	**1.45 (1.12; 1.87)**	460(54.0)	**2.89 (2.11; 3.94)**	195(66.8)	**2.25 (1.66; 3.04)**

*Adjusted by gender, age, maternal scholarity, parental asthma, mold, sewage system, *A.lumbricoides* and *T. trichiura* infections

Table 4: Association of *T. canis* infection with specific IgE for aeroallergens and skin prick test in 1148 studied children

***T.canis* infection (N=1148)**	***IgE (≥ 0.35)***			***IgE (≥ 0.70)***			***Skin prick test***		
	n (%)	Crude	Adjusted OR (95%C.I.)	n (%)	Crude	Adjusted OR (95%C.I.)	n (%)	Crude	Adjusted OR (95%C.I.)
Negative	269 (48.3)	1	1	209 (49.5)	1	1	205 (58.4)	1	1
Positive	288 (51.7)	**1.44 (1.14;1.82)**	**1.48 (1.15 1.91)**	213 (50.5)	1.24 (0.98;1.58)	**1.36 (1.04; 1.77)**	146 (41.6)	**0.73 (0.56;0.94)**	0.79 (0.60; 1.04)

*Adjusted by gender, age, maternal scholarity, parental asthma, mold, sewage system, *A.lumbricoides* and *T. trichiura* infections.

Tabela 5: Association of *T. canis* infection with specific IgE and skin prick test in 914 children non-infected by intestinal helminths

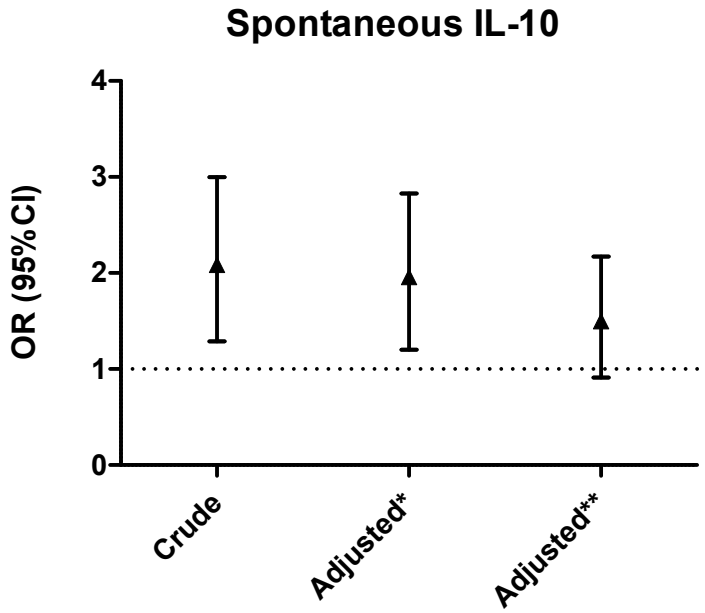
***T. canis* infection (N=914)**	***IgE ≥ 0.35 kU/L***		***IgE ≥ 0.70 kU/L***		***Skin prick test***	
	n (%)	Adjusted OR (95%CI)	n (%)	Adjusted OR (95%CI)	n (%)	Adjusted OR (95% CI)
Negative	191(35.4)	1	243(45.1)	1	187(34.7)	1
Positive	159(42.4)	**1.35 (1.2; 1.79)**	206(54.9)	**1.52 (1.15; 1.99)**	108(28.8)	**0.73 (0.54; 0.98)**

*Adjusted by gender, age, maternal sholarity, parental asthma, mold, sewage system.

Table 6: Polytomous analysis comparing non-atopic asthmatics and atopic asthmatics

Variable	*Asthma+IgE ≥0.70 (N=1148)*				
	Non-atopic asthmatic		*Atopic asthmatic1*		*Atopic asthmatic2*
	n(%)	Adjusted*	n(%)	Adjusted*	Adjusted*
T. canis					
Negative	69(49.6)	1	58(48.7)	1	1
Positive	70(50.4)	1.14 (0.76; 1.71)	61(51.3)	**1.62 (1.06; 2.48)**	1.12 (0.73; 1.72)

*Adjusted by Gender, Age, Maternal Scholarity, Parental Asthma, A.lumbricoides and T. trichiura.
1Subgroup of reference: non-atopic non-asthmatic
2Subgroup of reference: atopic non-asthmatic



5. DISCUSSÃO

A síndrome da *Larva Migrans Visceral* (LMV) é uma patologia de difícil diagnóstico clínico, principalmente por apresentar-se na maioria das vezes na forma assintomática ou com sinais clínicos bem diversificados (HARALAMBIDOU *et. al*., 2005; GAVIGNET *et. al*., 2008; SAPORITO *et. al*., 2008). Desta forma, o diagnóstico desta patologia é realizado quase que exclusivamente através da detecção de anticorpos IgG anti-*T.canis*.

Muitos laboratórios padronizaram ensaios *in house* para determinar a prevalência desta zoonose (AGUIAR-SANTOS et al, 2004). O antígeno ESLA do *Toxocara canis* obtidos em nosso laboratório possui bandas com peso molecular semelhantes as já padronizadas por outros autores (RUBINSKY-ELEFANT et. al., 2006; IDDAWELA et. al., 2007) o que permite a comparação dos nossos resultados com as prevalências obtidas em outros locais do mundo. A padronização do ensaio pelo nosso laboratório mostrou ser mais sensível quando comparado com a literatura, uma vez que, a diluição do soro foi de 1:1000 e do anticorpo conjugado 1:2000, com ponto de corte de 0,23. Outro ponto importante que pode ter influenciado nessa maior sensibilidade de nosso ensaio foi o fato de termos absorvido (pré-incubado) os soros com extrato de *A. lumbricoides* ao invés de *A. suum* como reportado na literatura na maioria dos trabalhos (LYNCH et. al., 1988; CAMARGO et al. 1992; NUNES et al. 1997; CAMPOS JUNIOR et. al., 2003; ROLDÁN et al. 2006). Acreditamos que a absorção com antígeno de *A. lumbricoides* é mais eficaz em bloquear os anticorpos que ligam-se cruzadamente por ser um parasito de humanos.

A absorção dos soros com antígeno de outros parasitos é uma prática que aumenta a especificidade do ensaio, uma vez que muitas espécies de parasitos compartilham antígenos

semelhantes o que ocasiona a reação cruzada entre estes anticorpos Os nossos resultados mostraram que a absorção com antígeno de *A.lumbricoides* diminuiu sensivelmente a Densidade Óptica (D.O.) dos soros, o que tornou o ensaio mais específico. Devido ao fato de encontrarmos freqüências moderadas de *A. lumbricoides* (14,9%) e *T. trichuira* (13,8%) nas crianças do estudo e os soros terem sido absorvido com extrato somático de *A. lumbricoides*, para evitar reação cruzada de IgG, acreditamos que nosso ensaio tem uma boa especificidade. Além disso, realizamos absorção de alguns soros com antígeno somático de *Trichuris trichiura* após a absorção com antígeno de *A. lumbricóides* e não observamos diminuição significativa na densidade óptica que justificasse a realização deste procedimento, principalmente devido à dificuldade de obtenção deste parasito. Os resultados mostraram quea pré-absorção com *A. lumbricóides* foi suficiente para evitar a reação cruzada com outros parasitos (dados não mostrados).

Nunes et al. (1997) determinaram pelo menos uma banda de peso molecular por volta de 55-66 kDa responsável pela reatividade cruzada entre *T. canis* e *A. suum*, e que esta desaparecia com a pré-absorção dos soros com antígeno de *A. suum*. Roldán e Espinoza (2009) determinaram bandas antigênicas reconhecidas por soros de pacientes com toxocaríase de 24, 28, 30, 35, 56, 117, 136 e 152 kDa. Contudo, somente as bandas de 24-35 kDa foram altamente específicas para infecção por Toxocara (98.3%), enquanto nas outras foram observadas reações cruzadas com soro de pacientes infectados com outros parasitos.

Em nosso estudo verificamos uma prevalência de IgG anti-*T.canis* de 48,4%. Won et. al. (2008) determinaram uma prevalência de 13,9% nos EUA, enquanto estudos conduzidos na América Latina indicaram uma soroprevalência semelhante aos diagnosticados em nosso trabalho. Alonso et. al. (2000) reportaram uma positividade de 37,9% em crianças menores de

14 anos na Argentina, Espinoza et. al. (2008) determinaram uma soropositividade de 32,4% no Peru e no Brasil, Coelho et. al (2004) diagnosticaram infecção em 38,3% das crianças estudadas. A soropositividade para *T.canis*, em geral, está associada à idade mais avançada das crianças. Em concordância com nossos resultados, Lescano et. al. (1998) também diagnosticou uma prevalência maior no grupo de crianças mais velhas. Entretanto, Radman et. al (2000) observaram maior percentagem de infectados em indivíduos mais novos. Enquanto que Alderete et.al. (2003) diagnosticaram uma soroprevalência de 38,8% em crianças com idade média de 9,4 anos. Tem sido demonstrado, portanto, que crianças com idade mais avançada são mais infectadas que crianças de menor idade, entretanto as referências ainda são pouco conclusivas e estes achados podem ser conseqüência de um efeito acumulativo, uma vez que o diagnóstico é sorológico e não se tem dados sobre a curva da queda de anticorpos IgG anti-*T. canis* em humanos.

A escolaridade materna foi utilizada neste trabalho como um indicativo da condição sócio-econômica da família. O resultado da soroprevalência de IgG anti-*T.canis* encontrado no presente trabalho assemelham-se aos observados em outras populações de baixa renda, onde a prevalência da infecção é maior dentre crianças de mães com menor nível de escolaridade. Estes dados são semelhantes aos determinados por Alderete et.al. (2003) onde a infecção por *Toxocara* foi inversamente proporcional a renda familiar.

O contato com cão tem sido apontado em diversos estudos como um importante fator de risco para a toxocaríase. Um estudo de corte transversal estimou uma freqüência de 52% de positividade para *T.canis* em 34 famílias no estado do Amazonas. Dos 55% dos indivíduos que tinham contato com cão em casa, 60% foram positivos (chi2= 14,317; p = 0,026), e dos que tinham contato com filhotes de cão em casa, 66,6% foram positivos (chi2= 22,149;

p=0,008), o que demonstra a associação entre o contato com o cão e a presença de anticorpos anti-*T.canis* (DAMIAN et. al*.,* 2007). Na Argentina, 100 indivíduos foram avaliados quanto à frequência de anticorpos anti-*T. canis* e 23% foram positivos, sendo que todos tinham contato com cão em casa (CHIODO *et. al*., 2006). Nossos resultados confirmam estes achados sendo a presença do cão em casa um fator de risco para infecção por *T.canis* nesta população de estudo (1.36 [1.07; 1.72])

Diversos trabalhos epidemiológicos apontam a contaminação do solo como determinante na infecção por *T. canis* . Diversos trabalhos no Brasil (GUIMARÃES *et. al*., 2005; ALMEIDA *et. al*., 2007; TYIO *et. al.,* 2008) e no mundo (MIZGAJSKA, 1997; DEVERA *et. al.,* 2008; MARTIN e DEMONTE, 2008) mostram que o solo de áreas públicas como praças, parques, campings e praia são importantes focos de transmissão. Em nosso trabalho não avaliamos a contaminação ambiental por ovos de *T.canis,* mas foi observado que a pavimentação da rua aumentou a chance de infecção o que nos faz supor que talvez os ovos de *T. canis* ficaram mais expostos facilitando a contaminação. Este fato pode ser explicado pelo fato dos ovos serem pequenos o suficiente para serem soterrados em áreas não pavimentadas dificultando a contaminação. Além disso, os gatos, que também foram um fator de risco neste estudo, possam estar contaminando o ambiente com ovos de *T. cati*, uma vez que com a pavimentação estes não podem enterrar sua fezes deixando-as expostas. Poucos estudos são realizados para estimar a infecção de gatos e seu potencial como causador da LMV. Martinez-Barbabosa et. al. (2003) determinaram, no México, uma prevalência de infecção de *T. cati* em gatos de 42,5%, o que nos faz pensar que este parasito possa ser freqüente em nosso meio e levanta a importância de maiores estudos a cerca da epidemiologia da LMV.

Alguns estudos referenciam que a contaminação do solo não é a única rota efetiva na toxocaríase humana e que os ovos de *T. canis* podem se embrionar no pêlo de cães, e o contato direto entre estes e humanos pode ser uma rota alternativa para explicar a epidemiologia da doença (WOLFE e WRIGHT, 2003). Aydenizoz-Ozkayhan *et. al*. (2008) coletou amostras de pêlo de 51 cães e analisou quanto à presença de ovos de *T.canis* e 21,56% dos cães tinham ovos nos seus pêlos. Roddie *et.al*. (2008) examinou 100 cães quanto à presença de ovos no pêlo através de lavagem. Foi encontrado ovos de *Toxocara* no pêlo de 67% dos cães, sendo que 95% das amostras eram provenientes de filhotes.

Adicionalmente, acreditamos que os ovos de *Toxocara sp.* podem ser carregados também em pêlo de gatos, o que pode explicar em parte a forte associação positiva entre a presença do gato e a infecção por *T. canis*, uma vez que a prevalência de gatos foi menor que a de cães e sua associação foi mais forte que a encontrada para presença de cães em casa. Portanto, este contato físico tão próximo entre os cães e gatos e seus proprietários possivelmente aumentam o risco de transmissão do *Toxocara*, e apontam para a necessidade de maior atenção ao risco potencial a que os humanos estão expostos (OVERGAAUW et al, 2009).

A infecção por *Toxocara* tem características peculiares. O parasito não completa seu ciclo evolutivo, permanecendo no estágio larval por tempo indeterminado no hospedeiro (MAGNAVAL et al, 2001). Apesar da dificuldade em diagnosticar clinicamente, sinais como marcada eosinofilia, aumento da IgE sérica e, em alguns casos, desordens pulmonares similares a asma podem ser observados no curso da doença (DESPOMMIER, 2003). Claramente este perfil imunológico é resultante do estímulo de células Th2, fenótipo este associado também à asma atópica e às alergias em geral. Além disso, tem sido observado em

camundongos infectados com *T.canis* aumento de atividade inflamatória e intensa migração eosinofílica para os pulmões (PECINALI et al, 2005).

Nossos dados sugerem que a infecção por *Toxocara* esta associada à maior predisposição a eosinofilia em ambos os pontes de corte >4% e >10%, mesmo quando ajustada para outras infecções helmínticas. Um estudo conduzido na Argentina determinou uma soroprevalência de 23% sendo que foi diagnosticado eosinofilia em 86,95% dos indivíduos positivos ($p < 0,001$, OR = 11,03) e todos tinham contato com cão em casa (CHIODO *et. al*., 2006). Ainda na Argentina, Martin *et. al.* (2008) determinou uma soropositividade em ELISA de 59% para *T. canis* sendo que foi mais freqüente na população soropositiva a eosinofilia (p=0,017) e as dificuldades respiratórias (p=0,05). Espinoza *et. al*. (2008) determinou uma freqüência de soropositividade para *T. canis* de 32,4%, sendo que das crianças positivas 77,96% tinham sintomas respiratórios, 61,02% manifestações oculares, 38,98% tinham sintomas hepáticos e 38,98% tinham eosinofilia leve a moderada.

Além da eosinofilia, outro sinal da infecção por *Toxocara* é o aumento de IgE sérica (BUIJS et al, 1997). Dessa forma, neste trabalho avaliamos a associação entre IgG anti-T. *canis* e IgE total e específica para aeroalérgenos. Com relação à IgE específica contra aeroalérgenos utilizamos dois pontos de corte, o primeiro ≥0.35 kU/L e o segundo ≥0.70 kU/L, sendo este mais representativo de atopia para nossa população de estudo.

O ponto de corte da IgE especifica contra aeroalérgenos diagnosticada pelo sistema de immunoCAP da Pharmacia é utilizada mundialmente é ≥0.35 kU/L, ponto este que refere-se ao nível detectável de IgE que está positivamente associada a atopia nos países desenvolvidos. Contudo, nos países tropicais e em desenvolvimento, onde há alta prevalência de parasitoses

intestinais, este ponto de corte não é o ideal, uma vez que os indivíduos possuem mais IgE circulante (BAQUEIRO, 2007, COOPER, 2008) e os médicos alergistas utilizam pontos de corte mais altos para associarem com atopia e manifestações alérgicas. Nossas análises mostraram que a infecção por *T.canis* associa-se com positividade a aeroalérgenos em ambos pontos de corte utilizados o que nos leva a hipotetizar a presença de reações cruzadas de IgE entre epítopos do *T. canis* e epítopos de alérgenos ambientais comuns como ácaros e baratas em ambos pontos de corte. Estas reações já foram descritas para antígenos de *A. lumbricoides* e ácaros (ACEVEDO et al, 2009) e entre *Anisakis simplex* e *T. canis* (PERTEGUER et al, 2003).

Por outro lado, nossos resultados mostraram que houve associação negativa entre teste cutâneo (SPT) positivo e infecção por *T. canis*, mas que esta não foi mantida quando ajustada para infecções para *Ascaris* e *Trichuris*. O ajuste realizado com as variáveis indicativas de outras infecções helmínticas mostra que estas podem ser, possivelmente, potenciais confundidoras. Entretanto a análise realizada nas crianças não infectadas com helmintos intestinais mostrou que infecção por *T. canis* está realmente associada com a diminuição da reatividade cutânea a aeroaléregnos. Este efeito sobre o SPT, ou seja, a ausência de reatividade cutânea apesar dos altos valores de IgE tem sido atribuída dois mecanismsos: a. a alta produção de IgE policlonal induzida pelo parasito que altera a fase efetora tardia da resposta Th2 alérgeno-específica saturando os mastócitos e impedindo a ligação cruzada das IgEs com os alérgenos, também conhecida como “hipótese do bloqueio da IgE” (HOLT et al, 1999; YAZDANBAKHAH et al, 2002) e b. a denominada “resposta Th2 modificada” induzindo alta produção de IL-10 que suscita a mudança de isótipo para IgG4. Esta imunoglobulina é reconhecida bloqueadora da reação alérgica, interagindo com os alérgenos e

prevenindo a ligação das IgEs presentes nos mastócitos aos mesmos, impedindo a liberação dos mediadores inflamatórios destas células (PLATTS-MILLS et al, 2001).

Por outro lado, a infecção por *T. canis* não foi associada à asma na análise multivariada (OR=1.17, 95%C.I.= 0.86;1.58) mas foi positivamente associada aos fenótipos de asmáticos atópicos na análise politômica, usando como referência as crianças não atópicas e não asmáticas (OR=1.62, 95%C.I.=1.06;2,48). Contudo, acreditamos que a análise utilizando como referência as crianças atópicas não asmáticas seria mais representativa para observarmos a asma atópica, uma vez que em muitas crianças ambos os fenótipos coexistem, ou seja, a asma atópica como classificada neste estudo poderia ser a coexistência da atopia e da asma e não exatamente asma atópica. Portanto os resultados da análise politômica utilizando o grupo de referência atópicos não asmáticos nos mostrou a infecção por *T. canis* associada à asma atópica e, portanto, essa associação positiva se deve somenta á associação com atopia.

Apesar de ter sido relatado na literatura a ação inflamatória pulmonar causada pelo *T. canis* em camundongos (ESPINOZA et al, 2002a,b), estes achados não foram observados em nosso estudo. Sabe-se que o *T. canis* induz diferentes padrões clínicos e que estes dependem do período de infecção (PECINALI et al, 2005). Acreditamos, portanto, que o "timing" da infecção pode explicar este fato, uma vez que, a minoria de crianças positivos em nossa população eram de infecções recentes, demonstrado pelo teste de avidez (3,3%). Pinelli et al (2007) demonstraram que o "timing" afetou a reatividade das vias aéreas e o número de eosinófilos no BAL de camundongos, sendo que a hiperreatividade só foi significativamente alta no início da infecção, já em relação à produção de IgE o oposto foi observado, sendo maior em infecções crônicas. Soma-se a isto o fato da infecção por *T. canis* estar

positivamente associada à produção espontânea de IL-10 em nossa população, o que nos leva a crer que a infecção crônica por *T. canis* tem uma capacidade imunomoduladora o que explica, em parte, a ausência de associação com asma e a negativação do SPT.

6. CONCLUSÔES

- A soroprevalência da infecção por *Toxocara canis* é alta na população de estudo, devido às baixas condições sócio-econômicas e ambientais.

- Os fatores de risco para a infecção por *Toxocara canis* estão intimamente relacionadas a presença da fonte transmissora (cão e gato) e principalmente ao baixo nível de educação sanitária da população.

- O *Toxocara canis* é um importante indutor do aumento de eosinófilos e IgE total sérica em indivíduos soropositivos para esta infecção.

- A infecção por *Toxocara canis* aumenta os níveis de IgE específica contra aeroalérgenos, aumentando, consequentemente, a atopia na população.

- A associação negativa entre a infecção pot *T. canis* e teste cutâneo deve-se possivelmente à indução policlonal de IgE dificultando a degranulação dos mastócitos sensibilizados com IgE anti-aeroalérgenos.

- A infecção crônica por *Toxocara canis* tem uma capacidade imunomoduladora, por induzir produção de IL-10, e pode ser um possível mecanismo da negativação do teste cutâneo.

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