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Investigação sorológica, molecular e de marcadores genéticos de histocompatibilidade, na população de uma região endêmica, para leishmaniose tegumentar americana no sul do Brasil

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito para obtenção do título de Doutor em Ciências da Saúde
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Orientador: Prof.ª Dr.ª Sueli Donizete Borelli

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COMISSÃO JULGADORA

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Universidade Estadual de Maringá (Presidente)

Profª. Drª. Thaís Gomes Verzignassi Silveira
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Universidade Estadual de Londrina

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Local de defesa: Sala 01, Bloco 126, *campus* da Universidade Estadual de Maringá.

DEDICATÓRIA

Dedico esta tese às pessoas mais importantes da minha vida, minha amada mãe Regina Celi Staniszewski, ao meu querido esposo Fabiano Rodrigo Silva e a minha doce filha Emanuela Ribas Silva.

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“Peça a Deus que abençoe os seus planos,
e eles darão certo”.
(Provérbios 16:3)

Investigação sorológica, molecular e de marcadores genéticos de histocompatibilidade, na população de uma região endêmica, para leishmaniose tegumentar americana no sul do Brasil

RESUMO

Neste estudo são relatados os resultados de uma investigação sorológica e molecular em indivíduos sem manifestações clínicas da leishmaniose tegumentar americana (LTA) e demonstrados os resultados da associação com marcadores genéticos HLA de Classe I (-A, -B, -C) e LTA em moradores de uma região endêmica do sul do Brasil. Foi realizado um estudo retrospectivo das fichas epidemiológicas de pacientes atendidos no Laboratório de Leishmanioses da Universidade Estadual de Maringá, para o diagnóstico de LTA, pertencentes a 13ª e 15ª Regional de Saúde do Estado do Paraná. Os pacientes que apresentaram positividade na pesquisa do parasito e/ou intradermorreação de Montenegro foram contatados para a coleta de material biológico e composição do grupo de casos. O grupo controle foi composto por indivíduos saudáveis com ausência de manifestações clínicas da doença e moradores de locais endêmicos para LTA. A detecção de anticorpos do grupo sem manifestação clínica da LTA foi realizada por *Enzyme-linked immunosorbent assay* (ELISA), e as amostras positivas, foram analisadas pela imunofluorescência indireta (IFI) para *Leishmania braziliensis* e *Trypanosoma cruzi.* A reação em cadeia da polimerase (PCR) foi realizada com os iniciadores MP3H/MP1L que amplificam fragmento do k-DNA de *Leishmania (Viannia)*. Todas as amostras do grupo de casos e controles foram submetidas à tipificação HLA-A, -B, -C pela metodologia PCR-SSO (tecnologia Luminex). Os resultados demonstraram a presença de infecção subclínica por *Leishmania* e o envolvimento do HLA-C*04 na susceptibilidade a LTA em moradores de uma região endêmica para LTA no sul do Brasil.

Palavras-chave: Leishmaniose. Susceptibilidade genética. HLA. ELISA. Imunofluorescência indireta. PCR.

Serological and molecular investigation and genetic markers histocompatibility, in individuals from an American cutaneous leishmaniasis-endemic region of southern Brazil.

ABSTRACT

We report the results of a serological and molecular research in individuals with no clinical manifestations of American cutaneous leishmaniasis (ACL) who lived in endemic area and we report the results of association between HLA class I genes (HLA-A, -B, and -C) and ACL in an endemic region of southern Brazil. A retrospective study was carried out on the epidemiological records of patients from the 13th and 15th Health Sections of the state of Paraná diagnosed with ACL. Patients with clinical manifestations of the disease and with positive parasite detection and/or Montenegro skin test were contacted for the collection of biological material and composition cases of group. The control group consisted of individuals with no clinical manifestations of ACL who lived in endemic region. Antibodies were detected in the healthy individuals by enzyme-linked immunosorbent assay (ELISA), and the positive samples were analyzed by indirect immunofluorescence (IIF) for *Leishmania braziliensis* and *Trypanosoma cruzi*. Polymerase chain reaction (PCR), employing the *MP3H*/*MP1L* primers, amplified a fragment of *Leishmania (Viannia)* k-DNA. All cases and controls group samples were subjected to HLA class I (HLA-A, -B, -C) typing were carried out through PCR-SSO using Luminex technology. The results reveal the presence of subclinical *Leishmania* infections and demonstrate the involvement of HLA-C*04 in the ACL susceptibility in individuals from an ACL-endemic region of southern Brazil.

***Keywords*:** Leishmaniasis. Genetic susceptibility. HLA. ELISA. Indirect immunofluorescence. PCR.

LISTA DE ILUSTRAÇÕES

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CAPÍTULO I

ASPECTOS GERAIS DA LEISHMANIOSE TEGUMENTAR AMERICANA

As leishmanioses representam um conjunto de enfermidades diferentes entre si, que dependendo da espécie de *Leishmania*, estado imunológico e composição genética do hospedeiro podem resultar em três formas clínicas diferentes: visceral, cutânea (Figura 1) e mucocutânea (Figura 2). Trata-se de uma doença infecciosa parasitária, causada por protozoários do gênero *Leishmania* que são transmitidos pela picada de flebotomíneos fêmeas infectadas (REY, 2008; CONDINO et al., 2008; BRASIL, 2010, WHO, 2015) .

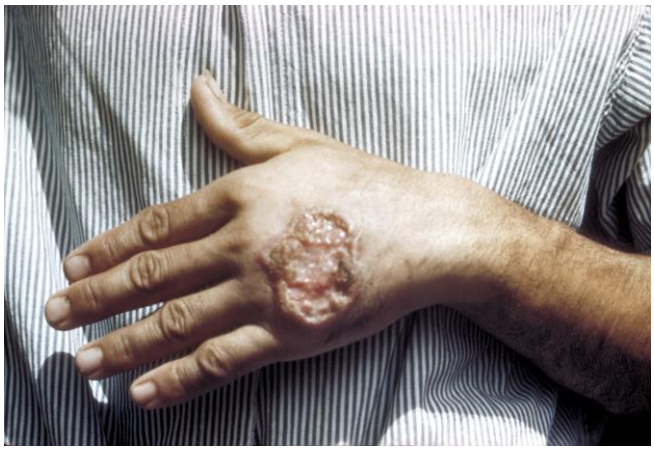

Figura 1. Leishmaniose cutânea.
Fonte: http://pt.wikipedia.org/wiki/Leishmaniose

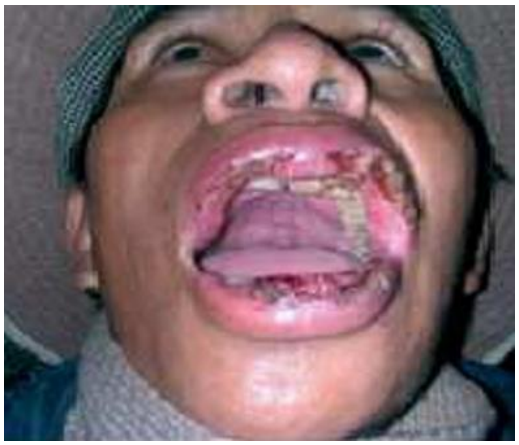

Figura 2. Leishmaniose mucocutânea.
Fonte: CHAPPUIS *et al* (2007).

A leishmaniose tegumentar americana (LTA) é um grupo de doenças de evolução crônica, provocada pela infecção das células do sistema fagocítico mononuclear por amastigotas de *Leishmania* spp., acometendo a pele e/ou mucosas, de forma localizada ou difusa (REITHINGER et al., 2007).

No Brasil, existem sete espécies de *Leishmania* que podem causar a LTA: *Leishmania (Leishmania) amazonensis, Leishmania (Viannia) guyanensis, Leishmania (Viannia) braziliensis, Leishmania (Viannia) lainsoni, Leishmania (Viannia) naiffi, Leishmania (Viannia) shawi and Leishmania (Leishmania) infantum* (LESSA et al., 2007; BRASIL, 2008). As espécies de *Leishmania* variam de acordo com a localização geográfica. Na região do Noroeste do Paraná, a espécie predominante (98,7%) é a *Leishmania (Viannia) braziliensis* (LONARDONI et al. 1993, SILVEIRA et al. 1999). A infecção por *L. braziliensis* pode evoluir para manifestação clínica na forma cutânea localizada e/ou formas mucosas (LONARDONI et al., 2006).

Existe ainda, outra forma descrita como subclínica ou assintomática que vem sendo detectada em moradores de áreas endêmicas (SAMPAIO et al., 2009; BRITO et al., 2008; ARRAES, et. al., 2008). Após a infecção por *Leishmania* spp., alguns indivíduos naturalmente resistentes apresentam uma resposta imunológica eficiente não apresentando manifestações clínicas (assintomáticos) (SILVEIRA et al. 1997; SILVEIRA et al. 2004).

EPIDEMIOLOGIA DA LTA

A epidemiologia da leishmaniose depende das características ecológicas dos locais de transmissão, das espécies de parasitos existentes em cada região e do histórico atual e passado da população em relação a exposição do parasito e o comportamento humano (WHO, 2015).

A leishmaniose é uma doença de repercussão mundial, ameaçando atualmente, 350 milhões de homens, mulheres e crianças em 88 países (WHO, 2015). Estima-se que ocorram 1,3 milhões de novos casos de leishmaniose anualmente, sendo que aproximadamente 20.000 a 30.000 evoluem para morte (WHO, 2015).

A leishmaniose tegumentar é a mais comum, sendo que aproximadamente 95% dos casos ocorrem nas Américas, na bacia do Mediterrâneo, no Oriente Médio e Ásia Central; mais de dois terços dos casos ocorrem em seis países: Afeganistão, Argélia, Brasil, Colômbia, Irã (República Islâmica) e a República Árabe da Síria. Estima-se que há 700 mil a 1,3 milhões de novos casos anuais no mundo (Figura 3). Em relação à leishmaniose mucocutânea, quase 90% dos casos ocorrem na Bolívia, Brasil e Peru (WHO, 2015).

No Brasil, a LTA é uma das zoonoses de maior importância clínica, tendo sido registrados 635.399 casos da doença entre 1990 e 2013. Destes, 13.889 são provenientes da região sul do país. O estado do Paraná contribui com a maior parte dos casos de LTA registrados na região sul, sendo 13.188 casos (94,9%) pertencem ao Estado do Paraná (BRASIL, 2014). Diversas cidades do noroeste do estado do Paraná possuem elevada incidência de casos de LTA caracterizando-se como uma região endêmica (CURTI et al., 2009; MONTEIRO et al., 2009).

Endemicidade da leishmaniose cutânea no mundo, 2013.

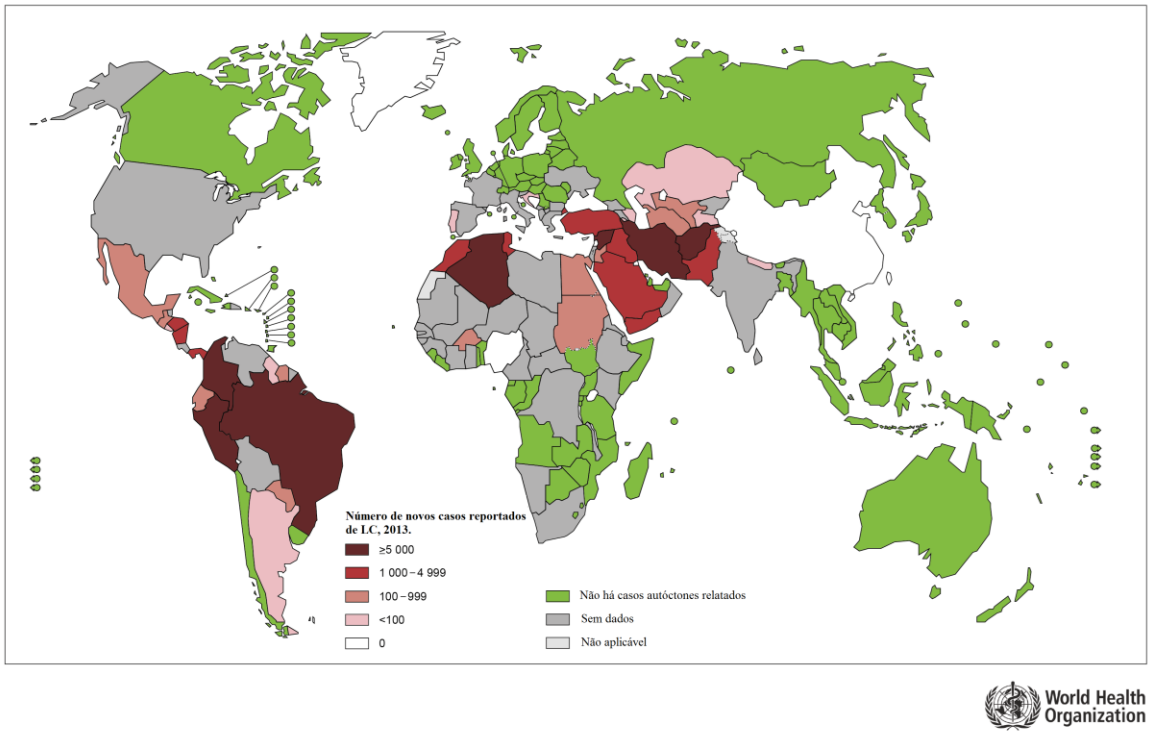

Figura 3. Situação da leishmaniose tegumentar no mundo.
Fonte: http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_CL.png (adaptado)

ANÁLISES SOROLÓGICAS E MOLECULARES NO DIAGNÓSTICO DA LTA

A utilização de métodos sorológicos e moleculares no diagnóstico da LTA auxilia não somente na confirmação dos achados clínicos, mas também pode fornecer importantes informações epidemiológicas, pela possibilidade de identificar as espécies circulantes de cada região, e isso, pode contribuir na elucidação das medidas a serem adotadas para o controle do agravo. (BRASIL, 2010).

Rotineiramente o diagnóstico laboratorial da LTA é realizado por meio da pesquisa direta do parasito, métodos imunológicos como a intradermorreação de Montenegro (IDRM) e testes sorológicos (BRASIL, 2010).

Em relação ao uso dos testes sorológicos, os mais utilizados para o diagnóstico da LTA são a aglutinação direta, imunofluorescência indireta (IFI) e ensaio imunoenzimático. A IFI é a mais empregada, porém com possibilidades de reações cruzadas, especialmente com a doença de Chagas (YONEYAMA et. al, 2007). Em pacientes com lesões entre um e seis meses de evolução é frequente a negatividade da IFI (SILVEIRA et. al., 1996). A associação

dos diferentes testes sorológicos pode aumentar a positividade da LTA no diagnóstico laboratorial (YONEYAMA et. al, 2007).

A técnica *Enzyme-linked immunosorbent assay* (ELISA) apresenta elevada sensibilidade (YONEYAMA et. al, 2007) e tem sido empregada para o diagnóstico de LTA, tanto em casos clínicos quanto em casos subclínicos (ARRAES et al., 2008; SZAGIKI et al., 2009). A utilização de diferentes antígenos em técnicas mais sensíveis, como a ELISA, pode fornecer diagnóstico mais específico (ARRAES et al., 2008).

Entre as análises moleculares, a reação em cadeia da polimerase (PCR) tem se mostrado uma boa ferramenta para o diagnóstico da LTA. Há vários iniciadores da PCR para a detecção de *Leishmania*, no entanto, estes não são suficientemente padronizados para uso generalizado, e portanto não disponibilizados na rotina laboratorial. (YONEYAMA et. al, 2007).

IMUNOPATOGENIA DA LTA

A LTA tem sido considerada uma das mais interessantes doenças causadas por parasitos em virtude da sua imnunopatogênese que envolve um complexo processo de interação entre a variedade de espécies de *Leishmania* que causam a doença e a resposta imunitária humana (SILVEIRA et al., 2009).

De maneira geral, a *Leishmania* spp. é um protozoário intracelular obrigatório que infecta macrófagos e pode causar uma doença inflamatória crônica envolvendo muitos tecidos (ABBAS E LICHTMAN, 2015). A infecção por *Leishmania*, em camundongos, tem auxiliado no estudo das funções efetoras de várias citocinas e dos subgrupos de linfócitos T auxiliares que as produzem (AGUILAR-TORRENTERA E CARLIER, 2001; HURDAVAL E BROMBACHER, 2014). Além disso, a resposta imunopatológica da LTA também tem sido estudada baseando-se principalmente nas células $TCD4^+$ e $TCD8^+$ e alguns perfis de citocinas produzidas por estas células nas lesões de pele e mucosas dos doentes (RIBEIRO-DE-JESUS et al. 1998; SILVEIRA et al., 2009; HURDAVAL E BROMBACHER, 2014).

Estudos evidenciam que se houver o predomínio das células Th1 nas lesões, estas células secretam a citocina IFN-γ e o indivíduo apresenta resistência à infecção (ROGERS et al., 2002; MURRAY et al., 2005). Em contraste, se as células Th2 são dominantes, estas promovem a produção de interleucina (IL) -4, IL-10 e outras citocinas que tornam o indivíduo suscetível e incapaz de controlar a infecção (ROMAGNANI, 1991; ROGERS et al., 2002).

COMPLEXO PRINCIPAL DE HISTOCOMPATIBILIDADE (CPH)

O Complexo Principal de Histocompatibilidade (CPH) ou *Major histocompatibility complex* (MHC), consiste em um conjunto de genes que está situado no braço curto do cromossomo 6 humano, na região p21.3 (SENGER et al., 1993). Muitos dos genes do CPH estão envolvidos em funções imunológicas. Em particular, o CPH contém um grupo de genes que codificam diversas proteínas que são expressas na superfície de vários tipos celulares. Nos seres humanos, tais moléculas são conhecidas como Antígenos Leucocitários Humano, ou sistema HLA, do inglês *Human Leucocyte Antigens* (PEAKMAN E VERGANI; 1999).

Os genes do HLA estão divididos em três grupos denominados de HLA Classe I, II e III (Figura 4). A região de HLA Classe III contém genes que codificam proteínas que não participam da apresentação de peptídeos aos linfócitos T (ABBAS E LICHTMAN, 2015).

A região do HLA Classe I contém genes denominados HLA-A, -B e -C, que codificam as moléculas clássicas de histocompatibilidade, assim denominadas por desempenharem a principal função no reconhecimento de peptídeos pelas células $TCD8^{+}$ (ABBAS E LICHTMAN, 2015). Estes genes codificam proteínas na superfície de praticamente todas as células nucleadas. Os genes HLA-E e F codificam moléculas encontradas apenas em tecidos fetais e em alguns tecidos da fase adulta, ao passo que os genes HLA-G codificam moléculas presentes apenas em tecidos placentários (COROSELLA et al., 1996). Os *loci* HLA-H, J, K e L não codificam proteínas, sendo denominados pseudogenes (ABBAS E LICHTMAN, 2015).

A região de HLA Classe II contém os genes denominados HLA-DR, -DQ e –DP. Os genes HLA-DR são subdivididos em DRA e DRB. Os genes HLA-DRB são subdivididos em DRB1 a 9. Os genes HLA-DQ e HLA-DP são todos polimórficos e são também subdivididos em DQA e DQB, e DPA e DPB. Os genes DQA e DPA codificam as cadeias α e os genes DQB e DPB codificam as cadeias β das moléculas de HLA-DQ e -DP, respectivamente (ABBAS E LICHTMAN, 2015).

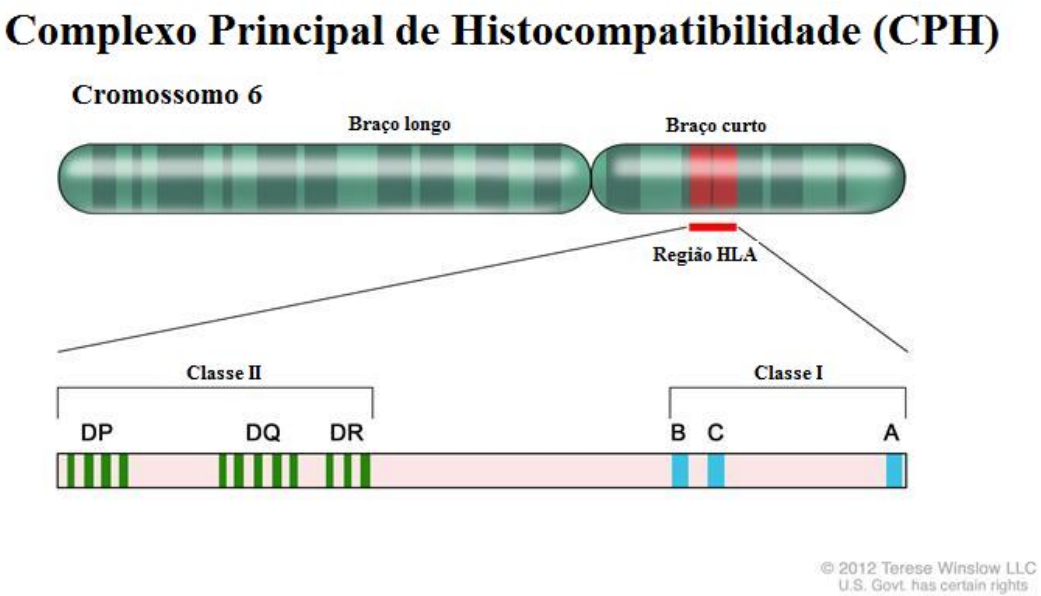

Figura 4. Estrutura genômica do complexo HLA.
Fonte: http://www.usp.br/aun/exibir.php?id=6088 (adaptado)

A participação do CPH nas respostas imunológicas a todos os antígenos protéicos evidenciam que os estudos sobre o CPH são importantes , sobretudo, em associação com doenças.

ASSOCIAÇÃO DOS GENES HLA COM LTA

Os antígenos HLA têm sido estudados em uma grande variedade de doenças de distintas etiologias, incluindo autoimunes, as infecciosas, as neoplásicas e as idiopáticas. Nos estudos populacionais, as frequências dos antígenos HLA, observadas em um grupo de pacientes não aparentados, são comparadas com aquelas de indivíduos-controle, também não aparentados (DONADI, 2000).

Em relação aos estudos de associação HLA e LTA, alguns grupos alélicos de classes I e II foram relatados na literatura, demonstrando estar envolvidos tanto na susceptibilidade como na proteção da doença (BARBIER et al., 1987; LARA et al., 1991; PETZL-ERLER et al., 1991; EL-MOGY, 1993; FAGHIRI et al., 1995; PROBST et al., 2000; MEDDE-GARNAOUI et al., 2001; OLIVO-DÍAZ et al., 2004; ALVES et al., 2006; SAKTHIANANDESWAREN et al., 2009; RIBAS-SILVA et al., 2013). A tabela 1 apresenta alguns estudos relacionados aos grupos alélicos HLA associados às diferentes espécies de *Leishmania*.

Tabela 1. Grupos alélicos HLA envolvidos na susceptibilidade e proteção em diferentes espécies de *Leishmania.*

Doença	*Leishmania spp*	País	HLA susceptibilidade	HLA proteção	Ref.
LV	*Leishmania Chagasi*	Nordeste, Brasil.	-	-	Peacock et al., 2002
LV	*Leishmania Infantum*	Irã	HLA-A26	-	Faghiri et al.,1995
LV	*Leishmania infantum*	Tunísia	-	HLADR2 HLADR13 HLA-DQB1	Medde-Garnaoui et al., 2001
LC	*Leishmania guyanensis*	Guiana Francesa	HLA- Cw7	-	Barbier et al.,1987
LC	*Leishmania braziliensis Leishmania guyanensis*	Venezuela	HLA-Bw 22 HLA-DQw3	-	Lara et al., 1991
LC	*Leishmania Mexicana*	México	HLA-DRB1 HLA-DQA1 HLA-DPA1 HLA-DPB1	HLA-DR2 HLA-DPB1	Olivo-Díaz et al., 2004
LC	*Leishmania braziliensis*	Noroeste do Paraná, Brasil.	-	HLA-B*45	Ribas-Silva et al., 2013
LCD	*Leishmania spp.*	Egito	HLA-A11 HLA-B7 HLA-B5	-	El-Mogy, 1993
LCM	*Leishmania braziliensis*	Noroeste do Paraná, Brasil	HLA- DQw3	HLA-DR2	Petzl-Erler et al., 1991

LC: Leishmaniose Cutânea; LCM: Leishmaniose Cutâneo-Mucosa; LCD: Leishmaniose Cutânea Difusa; LV: Leishmaniose Visceral.

TÉCNICAS UTILIZADAS PARA A TIPIFICAÇÃO HLA

A tipificação HLA pode ser determinada pelo método sorológico (TERASAKI E MCCLELLAND, 1964) e molecular (LAVANT et al. , 2011). As técnicas sorológicas utilizam antisoros específicos para identificar as moléculas HLA expressas nas superfícies celulares. No entanto, com o surgimento das técnicas moleculares, estas tornaram-se o método de escolha nos laboratórios de histocompatibilidade e de pesquisas científicas por proporcionar uma melhor resolução.

As técnicas moleculares mais utilizadas são a PCR - *Sequence Specific Primer* (PCR-SSP) e PCR - *Sequence Specific Oligonucleotide Probing* (PCR-SSOP). A PCR-SSP consiste primeiramente em amplificação do DNA genômico usando *primers* de sequência específica para cada grupo de alelos (baixa resolução) ou *primers de* sequência alelo-específica (alta resolução). Em seguida, realiza-se a eletroforese do material amplificado, posteriormente analisa a fotografia do gel e realiza-se a transferência dos resultados, observados no gel, para os *worksheets* (KIMURA E SASAZUKI, 1992; RIBAS-SILVA et al., 2014).

A técnica de PCR-SSOP consiste na amplificação do DNA genômico a partir de *primers* loco-específicos, seguida da imobilização do material amplificado em um suporte sólido, que posteriormente são hibridizadas com sondas alelo-específicas adequadamente marcadas (KIMURA E SASAZUKI, 1992).

Segundo Tan et al. (2000) a tipificação HLA pela metodologia SSP provou ser uma técnica precisa, confiável e reprodutível, porém a metodologia é considerada difícil para se adequar em larga escala e necessita, na maioria das vezes, de interpretações humanas, sem ajuda de *softwares*, aumentando assim a suscetibilidade a erros.

Embora na metodologia PCR-SSOP não seja descartada a hipótese de erros desta natureza, as metodologias SSO Dynal RELI™ e SSO Luminex™ possuem *softwares* próprios como ferramentas de auxílio às interpretações dos resultados. A disponibilidade de, no mínimo, duas metodologias para a tipificação das moléculas HLA são essenciais no esclarecimento de resultados duvidosos e também como controle de qualidade no laboratório de histocompatibilidade (RIBAS-SILVA et al., 2014).

JUSTIFICATIVA

A patogênese da leishmaniose, padrões clínicos e ciclo de vida do parasito já estão bem caracterizados. Da mesma forma, estudos sobre as respostas imunológicas evidenciam que as células Th2 dominantes promovem a produção de interleucina (IL) IL-4, IL-10 e outras citocinas que tornam o indivíduo suscetível e incapaz de controlar a infecção (ROMAGNANI, 1991; ROGERS et al., 2002). Em contraste, se as células Th1 prevalecerem, secretam a citocina IFN-ɤ e o indivíduo apresenta boa resposta à infecção (ROGERS et al., 2002; MURRAY et al., 2005). Entretanto, para o melhor entendimento das diferentes evoluções clínicas entre cada hospedeiro imunocompetente infectado pela mesma espécie de parasito, outros estudos são necessários, dentre eles, estudos relacionados com marcadores genéticos do CPH.

O HLA está intimamente ligado às respostas imunológicas devido ao fato deste apresentar antígenos às células de defesa. Assim, os alelos HLA de classes I e II têm sido associados com a susceptibilidade, proteção e manifestação clínica de várias doenças, inclusive em infecções por *Leishmania spp.* (BARBIER et al., 1987; LARA et al., 1991; PETZL-ERLER et al., 1991; EL-MOGY, 1993; FAGHIRI et al.,1995; PROBST et al., 2000; MEDDE-GARNAOUI et al., 2001; OLIVO-DÍAZ et al., 2004; ALVES et al., 2006; SAKTHIANANDESWAREN et al., 2009; RIBAS-SILVA et al., 2013). Na região sul do Brasil os estudos de associação HLA e LTA são escassos. Constatamos um estudo com estes marcadores genéticos associados à LTA na forma mucosa, onde os pesquisadores utilizaram o método sorológico para a tipificação HLA (PETZL-ERLER et al., 1991).

Recentemente conduzimos uma pesquisa, na região noroeste do estado do Paraná, de associação HLA e LTA cutânea. A metodologia utilizada para a definição deste marcador foi o método molecular. Até onde sabemos este trabalho foi pioneiro neste estudo de associação na região e na metodologia utilizada para a tipificação das moléculas HLA (RIBAS-SILVA et al., 2013).

O complexo processo de interação entre a variedade de espécies de *Leishmania* e os mecanismos da resposta imune na LTA ainda não estão devidamente esclarecidos. A escassez de trabalhos envolvendo os genes HLA e leishmanioses, sobretudo na região sul do Brasil, justifica-se a identificação destas moléculas nos diferentes espectros clínicos da LTA. Os dados obtidos poderão fornecer informações sobre os fatores genéticos do hospedeiro relacionados à resistência, à susceptibilidade e à progressão da doença contribuindo na definição de novos alvos para a terapia imunomoduladora.

No decorrer das análises sorológicas do grupo controle (indivíduos saudáveis e moradores de áreas endêmicas) pelo método ELISA-*Leishmania*, observou-se positividade em algumas amostras. Diante desta constatação, além do estudo de associação HLA e LTA, foi elaborado também um artigo sobre a investigação sorológica e molecular da LTA em indivíduos saudáveis de uma região endêmica.

OBJETIVOS

GERAL

Investigar a presença de infecção subclínica da LTA na população de uma região endêmica e verificar associação dos marcadores genéticos HLA com a LTA.

ESPECÍFICOS

Identificar e localizar os pacientes cadastrados no LEPAC/UEM, 13ª e 15ª Regional de Saúde do Estado do Paraná que tiveram LTA no período de 2000 a 2009.

Realizar os testes sorológicos e moleculares para detecção de infecção subclínica para a LTA.

Realizar os testes para detecção dos marcadores genéticos HLA de Classe I (-A, -B, -C) no grupo de pacientes e controles.

Analisar as correlações entre os marcadores genéticos do sistema HLA de Classe I e a LTA.

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CAPÍTULO II

Artigo 1: “ASSOCIATION BETWEEN HLA-C*04 AND AMERICAN CUTANEOUS LEISHMANIASIS IN ENDEMIC REGION OF SOUTHERN BRAZIL”

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ASSOCIATION BETWEEN HLA-C*04 AND AMERICAN CUTANEOUS LEISHMANIASIS IN ENDEMIC REGION OF SOUTHERN BRAZIL.

HLA-C*04 and American cutaneous leishmaniasis

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ABSTRACT

Leishmaniasis is a parasitic infectious disease with global repercussions. American Cutaneous Leishmaniasis (ACL) is endemic in southern Brazil and its pathogenesis varies according to parasite species, immune response, and host genetics. In terms of immunogenetics, many host genes including HLA (human leukocyte antigen), may be involved in susceptibility to and protection against ACL. In this context, the aim of this study was to investigate an association between HLA class I genes (HLA-A, -B, and -C) and ACL in an endemic region of southern Brazil. The allele frequencies of 186 patients diagnosed with ACL and 278 healthy individuals was compared. HLA class I (HLA-A, -B, and -C) typing were carried out through PCR-SSO using Luminex technology. These results reveal an association between the HLA-C*04 allele and the patient study group, in which it appeared more frequently than in the control group [21.5% vs. 13.49% (P = 0.0016 and Pc = 0.0258; OR=1.7560; CI 95%= 1.2227 – 2.5240)], thereby suggesting an increased susceptibility to ACL. Additional allelic groups such as HLA-A*02, HLA-B*35, HLA-B*45, HLA-C*01, and HLA-C*15 were also implicated however, further investigation is necessary to confirm their association with ACL. Therefore, the results obtained in this study demonstrate the involvement of HLA class I genes in the susceptibility or resistance particularly to ACL, with significant association between HLA-C*04 and ACL susceptibility.

Keywords: Genetic susceptibility; HLA; Leishmaniasis.

INTRODUCTION

Leishmaniasis is a parasitic infectious disease caused by protozoa of the genus *Leishmania* that are transmitted through the bite of infected sand flies. Depending on the species of *Leishmania*, the immunological status, and genetic composition of the host, the infection can result in four different clinical manifestations: cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Rey, 2008; Condino et al., 2008; Brasil, 2015)
In Brazil, cases of American Cutaneous Leishmaniasis (ACL) have been reported since the 1980s. Between 1990 and 2013, 635,399 cases of ACL were reported, out of which 13,889 occurred in southern Brazil and of those 13,188 (94.9%) occurred in the state of Paraná (Brasil, 2015). The northern and western regions of the state of Paraná were considered to be of epidemiological importance due to the largest number of cases occurring in those areas (Lima et al., 2002). In southern Brazil, the predominant species *Leishmania (Viannia) braziliensis* (98.7%) is responsible for the development of the two main forms of leishmaniasis, CL and MCL (Lonardoni et al., 1993; Silveira et al., 1999).
The pathogenesis, clinical patterns, and life cycle of the leishmaniasis parasite have already been well characterized. As such, immunological studies have shown that when activated T helper (Th) cells such as Th2 are dominant, production of interleukin (IL) -4, IL-10, and other cytokines are promoted and render the individual more susceptible and unable to control the infection (Romagnani, 1991; Rogers et al., 2002). Alternatively, when Th1 cells prevail, cytokine IFN-ɤ is secreted, and the individual becomes resistant to the infection (Rogers et al., 2002; Murray et al., 2005). However, larger immunogenetic studies such as investigations related to the genetic markers of major histocompatibility complex (MHC) are still required to better understand the reason for different clinical outcomes among immunocompetent hosts infected by the same parasite species.
MHC is the set of genes responsible for encoding histocompatibility molecules, which in humans are known as human leukocyte antigen (HLA) (Ellis and Ballingall, 1999). HLA is closely linked to the activation of the immune response, as it encodes antigen- presenting proteins to alert immune cells. As such, HLA class I and II alleles have been associated with susceptibility, protection, and clinical manifestations of various diseases including infection caused by *Leishmania spp*.(Barbier et al., 1987; Lara et al., 1991; Petzl-Erler et al., 1991; Probst et al., 2000; Alves et al., 2006; Sakthianandeswaren et al., 2009). However, these sorts of genetic studies are uncommon in southern Brazil, where to date only one small study was done to assess a few genetic markers (Petzl-Erler et al., 1991).
In this context, the aim of this study was to investigate the possible involvement of HLA class I genes (HLA-A, -B, -C) in the susceptibility to and protection from ACL among individuals from an endemic region of southern Brazil.

MATERIALS AND METHODS

Patients and controls

A retrospective study was conducted using epidemiological records of patients, from the 13th and 15th Regional Health District of the State of Paraná, who were screened and diagnosed with ACL in the Leishmaniasis Laboratory at the State University of Maringá. To proceed with this investigation, we selected patients that tested positive to the parasite and/or Montenegro's intradermal reaction.
The study population was formed by a group of 186 patients who were positively diagnosed with ACL and who received prescription medication. The control group was composed of 278 healthy individuals who were matched according to their age, gender, ethnicity, occupation,

and other demographic characteristics, presenting no clinical manifestations of the disease and that were living in an ACL endemic area. Individuals belonging to the two study groups had no family relation.
During the time of data collection, the patients ages ranged from 17 to 83 years old (average age of 47.06 ± 14.52 years), 139 (82.3%) were male and 30 (17.7%) were female. Regarding ethnicity of the patients, 140 (75.3%) were caucasian, 40 (21.5%) amerindians, 4 (2.2%) african, and 2 (1.0%) asian.
The ages of control/ healthy individuals ranged from 18 to 73 years old (average age of 33.5 ± 11.28), 222 (79.9%) were male and 56 (20.1%) were female. The ethnic distribution consisted of 166 (59.7%) caucasian, 28 (10.1%) amerindians, 11 (4.0%) african, 8 (2.9%) asian, and 65 chose not to inform (23.3%).
The individuals involved in this study were interviewed and data was collected and recorded in socio-epidemiological reports, which included information concerning clinical manifestations presented by the patient after treatment, such as development of the mucosal form of the disease, or even disease recurrence. All of the patients were informed about the study to be conducted and were required to sign a consent form upon agreement for participation. This study was approved by the Standing Committee on Ethics in Research Involving Human Beings at the State University of Maringá, according to protocol No. 153/2009.

Determination of HLA alleles
Samples of peripheral blood (10 mL) were collected from all participants (patients and controls) in tubes containing the anticoagulant EDTA, and centrifuged at 960*g* for 10 minutes. Nucleated cells were separated and frozen at- 80ºC until further analysis. Genomic DNA was extracted from100µL of frozen cells using the EZ-DNA extraction kit (Biological Industries®, Kibbutz Beit Haemek, Israel) following the manufacturer's instructions. The purity and concentration of DNA was determined using a NanoDrop 2000c/2000 UV-Vis spectrophotometer.
HLA class I genotypes (A, B, and C) of patients and controls were analyzed by PCR-SSO (polymerase chain reaction –sequence specific oligonucleotide) with Luminex technology (One Lambda Inc., Canoga Park, CA, USA) in the Laboratory of Immunogenetics at the State University of Maringá.

Statistical analysis
HLA-A, -B and -C genotyping results were tabulated using a database created in Excel 2007.The results were expressed as allele frequency (*fa%*), calculated by dividing the number of times different alleles appeared in the sample by the total number of alleles. The p value was calculated by two-tailed Fisher's exact test and the adjusted p value was calculated using Bonferroni correction adjusted for each *locus* for multiple comparisons. The *odds ratio* (OR) was calculated with a confidence interval (CI) of 95% for p values < 0.05. Descriptive statistical analysis for gender and ethnicity variables was calculated by Fisher's exact test, whereas the age variable was calculated by t test for independent samples. Statistical analyses for all tests were obtained using the R program set at a significance level of 5%. The Hardy-Weinberg (HW) equilibrium was analyzed using the Arlequin v. 2000 program (http:// anthropologie.unige.ch/arlequin/).

RESULTS
In this study, a significant difference was observed between patients with ACL and healthy individuals in regards to the presence of the HLA-C*04 allele. This allele appeared with

greater frequency in patients [21.5% vs. 13.5% (*P* = 0.0016 and *Pc* = 0.0258; OR= 1.7560; CI 95% = 1.2227 – 2.5240)], thereby suggesting an increased susceptibility to ACL as compared to healthy individuals (Table 1). Also noteworthy, is that HLA-C*04,04 (homozygous) patients are more susceptible to ACL than heterozygous patients (*P* = 0.0206; OR= 3.4227; CI 95% 1.0740 – 12.7812).

The possible involvement of ACL and HLA class I was also observed for the following allelic groups: HLA-A* 02 [27.0% vs. 34.0% (*P* = 0.0301 and *Pc* = 0.4405; OR= 0.7239; CI 95% = 0.5361 – 0.9743)], HLA-B*35 [16.4% vs. 10.6% (*P* = 0.0124 and *P*c = 0.3118; OR= 1.6512; CI 95% = 1.1028 – 2.4744)], HLA-B*45 [0.0% vs. 1.8% (*P* = 0.0073 and *P*c = 0.1973)], HLA-C*01 [5.1% vs. 2.2% (*P* = 0.0237 and *P*c = 0.3185; OR = 2.4376; CI 95% = 1.1076 – 5.5792)], HLA-C*15 [2.7% vs. 6.7% (*P* = 0.0087 and *P*c = 0.1305; OR= 0.3878; CI 95% = 0.1697 – 0.8081)]. Although these values were significant by the Fisher's exact test, no statistical significance was observed upon Bonferroni correction (Table 1). Among these allelic groups, evidence was found in the homozygous analysis of HLA-B* 35, 35, in which 7 individuals were identified in the patient study group compared to only 1 in the control group (*P* = 0.0081; OR = 10.784; CI 95% 1.3671 – 4.88,7544).

DISCUSSION

To investigate whether genetic factors within the MHC gene cluster region may contribute to susceptibility and/or protection against ACL, the allele frequencies of HLA class I were compared between two groups, ACL-positive patients and healthy individuals. The values obtained for the HLA-C*04 allele (*P* = 0.0016) were significant, with the greatest allele frequency occurring in the patient study group (21.5% vs. 13.5%). These results suggest a possible involvement of HLA-C*04 in ACL susceptibility.

Lara and collaborators (Lara et al., 1991) previously reported the involvement of the HLA-Cw4 allele (*P* = 0.0095) in ACL in a serological study conducted among family members in Venezuela. Our findings also support the association of ACL with the HLA-C*04 allelic group and to further emphasize this association, significance (*Pc* = 0.0258) was determined by Bonferroni correction. Findings in the literature also report a correlation of the HLA-C (HLA-Cw7) *locus* in the pathogenesis of cutaneous leishmaniasis (Barbier et al., 1987; Lara et al., 1991).

Furthermore in this study, allelic groups HLA-A*02, HLA-B*45, and HLA-C*15 were also considered potential factors of resistance to ACL. Moreover, significant p value determined by Fisher's exact test suggests possible involvement in disease susceptibility for HLA-B*35 and HLA-C*01. However, additional studies are required to confirm the conclusion drawn from these results. It is important to notice that although in this study the p value for the HLA-B*45 allelic group (*P* = 0.0073; *Pc* = 0.1973) was not significant after Bonferroni correction, its involvement in ACL should still be considered because this allele is not very common among the Brazilian population (Allele Frequencies, 2015). In addition, we reported a tendency toward ACL resistance for HLA-B*45 (*P* = 0.0046; *Pc* = 0.1426) in a previous study conducted with individuals from the same geographic region, compared to a different control group (RIBAS-SILVA et. al., 2013). Furthermore, it is possible to affirm that this finding is not due to ethnic differences between patients and healthy individuals. While no one in the patient study group expressed the HLA-B*45 allele, 10 control group individuals who were 60% caucasian, 10% african, 10% amerindians, and 20% not identified, did express the HLA-B*45 allele.

The population of southern Brazil is heterogeneous in its racial composition and presents a high degree of miscegenation, consisting primarily of caucasian descendants of europeans, africans and native americans. According to the phenotypic classification system of HLA

(Probst et al., 2000), the predominant population of the region selected for this study was of european origin (80.6%), with a smaller contribution of africans (12.5%) and native americans (7.0%). Considering this fact, our study also compared the results obtained for individuals of the patient study group and the control group for those who were reported to be caucasian/white. The results for this comparison remained significant for implicating the HLA-C*04 allele (*P*= 0.0021 and *P*c= 0.0290) in the development of ACL (data not shown). Petzl-Erler et al. (1991) also demonstrated the involvement of HLA class I in ACL in a different region of southern Brazil. These authors compared 43 subjects diagnosed with mucosal leishmaniasis to 111 healthy control individuals where detection of the HLA-B27 antigen (*P* = 0.029) was initially significant but, not significant after determination of the p value. The findings of our study corroborates the involvement of the HLA-B*27 allelic group in 15 patients who suffered from mucosal leishmaniasis (*P* = 0.0057 and *Pc* = 0.1578; OR= 12.0994; CI 95% = 1.7872 – 66.0541) (data not shown).

By analyzing the distribution of allele frequencies using the Arlequin v. 2000 program (http:// anthropologie.unige.ch/arlequin/), it was observed that in the ACL patient group, HLA-A (*P* = 0.0245) and HLA-C (*P* = 0.0311) had p values < 0.05, whereas for the other alleles observed in the case group and all control groups, we considered Hardy-Weinberg (HW) equilibrium at (*P* > 0.05). Although there was a shift in HW equilibrium, the data obtained in this study supports the potential role for specific HLA alleles in susceptibility or resistance to ACL. The association between ACL and the C *locus* and/or homozygosity may have influenced this deviation.

Some studies have shown an association of HLA class II with ACL (Petzl-Erler et al., 1991; Olivo-Díaz et al., 2004; Ribas-Silva et al., 2013). Considering this, future studies should also be performed using markers for HLA class II in southern Brazil.

We believe in the importance of developing new studies related to these and other genetic markers, investigating localized populations and analyzing allelic diversity, with the aim of identifying new associations or strengthening those that already exists. This knowledge will contribute to future prophylactic or therapeutic interventions in Brazilians at high risk for contracting american cutaneous leishmaniasis.

CONCLUSION

These results implicate the involvement of the HLA class I gene in the susceptibility to and/or protection from ACL. Although, we have not confirmed the involvement of some of the alleles implicated, a significant association was observed between HLA-C* 04 and ACL. In addition, this particular information was further supported by Bonferroni correction, which enables us to believe the influence of this allele on ACL infection in the endemic regions of southern Brazil.

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Table 1. Frequencies of HLA-A, -B and -C alleles in American cutaneous leishmaniasis (N=186) *versus* controls(N=278).

	ACL (N=186)		Controls (N=278)								ACL (N=186)		Controls (N=278)						
Allele	**n**	***fa* %**	**n**	***fa%***	**p-value**	**Pc-value**	**OR**	**CI 95%**		**Allele**	**N**	***fa%***	**N**	***fa%***	**p - value**	**Pc - value**	**OR**	**CI 95%**	
HLA – A*										**HLA-B***									
01	41	11.0	52	9.4	ns					**42**	3	0.8	4	0.7	ns				
02	101	27.2	189	34.0	0.0301	0.4405	0.7239	0.5361	0.9743	**44**	46	12.4	61	11.0	ns				
03	32	8.6	59	10.6	ns					**45**	0	0.0	10	1.8	0.0073	0.1973	-	-	-
11	27	7.3	25	4.5	ns					**47**	0	0.0	1	0.2	ns				
23	18	4.8	26	4.7	ns					**48**	3	0.8	0	0.0	ns				
24	43	11.6	55	9.9	ns					**49**	9	2.4	9	1.6	ns				
25	4	1.1	5	0.9	ns					**50**	10	2.7	20	3.6	ns				
26	12	3.2	15	2.7	ns					**51**	33	8.9	63	11.3	ns				
29	10	2.7	20	3.6	ns					**52**	12	3.2	12	2.2	ns				
30	16	4.3	18	3.2	ns					**53**	7	1.9	5	0.9	ns				
31	20	5.4	26	4.7	ns					**54**	1	0.3	1	0.2	ns				
32	10	2.7	16	2.9	ns					**55**	8	2.2	4	0.7	ns				
33	5	1.3	15	2.7	ns					**56**	2	0.5	1	0.2	ns				
34	3	0.8	2	0.4	ns					**57**	8	2.2	15	2.7	ns				
36	2	0.5	0	0.0	ns					**58**	6	1.6	10	1.8	ns				
66	4	1.1	3	0.5	ns					**59**	0	0.0	1	0.2	ns				
68	20	5.4	25	4.5	ns					**81**	0	0.0	1	0.2	ns				
74	4	1.1	5	0.9	ns					**HLA – C***									
HLA-B*										**01**	19	5.1	12	2.2	0.0237	0.3185	2.4376	1.1076	5.5792
07	23	62	46	8.3	ns					**02**	23	6.2	28	5.0	ns	ns			
08	16	4.3	36	6.5	ns					**03**	45	12.1	55	9.9	ns	ns			

13	1	0.3	10	1.8	ns					**04**	80	21.5	75	13.5	0.0016	0.0258	1.7560	1.2227	2.5240
14	17	4.6	34	6.1	ns					**05**	17	4.6	28	5.0	ns	ns			
15	30	8.1	50	9.0	ns					**06**	24	6.5	48	8.6	ns	ns			
18	20	5.4	29	5.2	ns					**07**	73	19.6	134	24.1	ns	ns			
27	7	1.9	5	0.9	ns					**08**	15	4.0	33	5.9	ns	ns			
35	61	16.4	59	10.6	0.0124	0.3118	1.6512	1.1028	2.4744	**12**	26	7.0	51	9.2	ns	ns			
37	1	0.3	5	0.9	ns					**14**	10	2.7	14	2.5	ns	ns			
38	10	2.7	15	2.7	ns					**15**	10	2.7	37	6.7	0.0087	0.1305	0.3878	0.1697	0.8081
39	16	4.3	22	4.0	ns					**16**	20	5.4	30	5.4	ns	ns			
40	19	5.1	23	4.1	ns					**17**	8	2.2	9	1.6	ns	ns			
41	3	0.8	4	0.7	ns					**18**	2	0.5	2	0.4	ns	ns			

N = number of times that this allele appeared; af = allelic frequency; P-value = calculated by the Fisher's exact test; Pc-value = p value adjusted by Bonferroni correction; OR = odds ratio; CI (95%) = 95% Confidence Interval; ns= not significant (p>0.05).

Artigo 2: “SEROLOGICAL AND MOLECULAR INVESTIGATION OF CUTANEOUS LEISHMANIASIS IN HEALTHY INDIVIDUALS FROM AN AMERICAN CUTANEOUS LEISHMANIASIS-ENDEMIC REGION”

American Journal of Infectious Diseases

Original Research Paper

Serological and molecular investigation of Cutaneous Leishmaniasis in Healthy Individuals from an American Cutaneous Leishmaniasis-Endemic Region

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Abstract American cutaneous leishmaniasis (ACL) is a major, clinically relevant zoonosis in Brazil. The disease spectrum includes single, localized, cutaneous ulcers, diffuse cutaneous leishmaniasis, and mucosal disease. A subclinical form of the disease has also been described in individuals living in ACL-endemic regions. The goal of this study was to employ immunological and molecular diagnostic methods to evaluate the presence of subclinical ACL in healthy individuals from an ACL-endemic region in northwestern Paraná. Antibodies IgG were detected by enzyme-linked immunosorbent assay (ELISA), and the positive samples were analyzed by indirect immunofluorescence (IIF) for *Leishmania braziliensis* and *Trypanosoma cruzi*. Polymerase chain reaction (PCR), employing the *MP3H*/*MP1L* primers, amplified a fragment of *Leishmania (Viannia)* k-DNA. Of the 159 individuals analyzed, 31 presented ELISA-positive serology, and 5 and 8 of these were IIF-positive for *Leishmania* and *T. cruzi*, respectively. All 159 individuals were PCR-negative. Most ELISA-positive individuals were males, and the cities São Jorge do Ivaí and Doutor Camargo showed the highest prevalence of positive individuals. Our results reveal the presence of subclinical *Leishmania* infections in inhabitants of this region. Further investigation of this population may contribute to understanding the immune responses to ACL.

Keywords: Leishmaniasis; subclinical cases; ELISA; polymerase chain reaction; indirect immunofluorescence

Introduction

American cutaneous leishmaniasis (ACL) is clinically one of the most important zoonoses in Brazil, with 635,399 recorded cases between 1990 and 2013 (Brasil, 2009; Brasil, 2015). A majority of the ACL cases recorded in southern Brazil originated in the state of Paraná. Data obtained from the Health Surveillance Secretariat revealed that 13,899 disease cases were recorded in southern Brazil between 1990 and 2013, 13,188 of which were from Paraná (Brasil, 2015). Several northwestern cities in the state of Paraná have shown a high incidence of ACL; therefore, it is an endemic region for this disease (Curti *et al.*, 2009; Monteiro *et al.*, 2009). *Leishmania braziliensis* is the main etiological agent of ACL in the state of Paraná (Lonardoni *et al.*, 2006; Szargiki *et al.*, 2009). The disease spectrum includes single, localized, cutaneous ulcers, diffuse cutaneous leishmaniasis, and mucosal disease. A subclinical or asymptomatic form of this disease has also been observed in individuals living in the ACL-endemic regions (Sampaio *et al.*, 2009; Brito *et al.*, 2008; Arraes, *et. al.*, 2008).

ACL is routinely detected in the laboratory by the direct detection of the parasite and immunological methods, such as the Montenegro skin test (MST) and indirect immunofluorescence (IIF) (Brasil, 2009). Enzyme-linked immunosorbent assay (ELISA)-based methods of diagnosis are known to be highly sensitive (Yoneyama, 2007), and have been employed in the diagnosis of both clinical and subclinical cases of ACL (Arraes *et al.*, 2008; Szagiki *et al.*, 2009). Previous

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reports have also confirmed the applicability of polymerase chain reaction (PCR) in the diagnosis of ACL, although it is not routinely used for this purpose (Yoneyama, 2007).

The aim of this study was to employ immunological and molecular diagnostic methods to evaluate the presence of subclinical ACL in healthy individuals native to an ACL-endemic region in northwestern Paraná.

Material and Methods

Study Design

A retrospective study was conducted using the epidemiological records of patients between the years 2005 and 2011, assisted by the Laboratório de Ensino e Pesquisa da Universidade Estadual de Maringá, Paraná (LEPAC/UEM), a Reference Center of the Ministry of Health for laboratory-based diagnosis of ACL. Individuals with ACL were identified from these records, and grouped according to the municipality of their residence. Relatives and neighbors living within a 1 km radius of these patients, or individuals who frequently visited ACL-endemic areas (woods and rivers), aged 18 years or above, were invited to take part in this study. Blood samples were collected from these subjects (n = 159) for ELISA-IgG and PCR analyses. ELISA-positive samples were subsequently analyzed for *T. cruzi* and *Leishmania braziliensis* antibodies by IIF.

Sample preparation and storage

A 10 mL aliquot of the blood sample was collected and split in two 5 mL tubes, one containing ethylenediaminetetraaceticacid (EDTA) and one without an anti-coagulating agent. Serum and buffy coat fractions were obtained by centrifuging the blood samples at 3,000 rpm for 15 minutes, and stored at -30°C until further use.

ELISA

Promastigote forms of *L. braziliensis* (MHOM/BR/1987/M11272) were cultured at 25°C in 199 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) inactivated bovine fetal serum, penicillin G (100 UI/mL), streptomycin (100 µg/mL), and 1% (v/v) human urine. Parasites were washed in phosphate buffered saline (PBS; pH 7.2), centrifuged at 4°C and 1,700 × *g* for 10 minutes, lyophilized, and stored at 4°C. The antigens were prepared according to the protocol described by Yoneyama et al. (2007). Briefly, the plates were sensitized with a dilution of the extract in carbonate-bicarbonate buffer (0.1 M, pH 9.6); the serum samples were diluted to a rate of 1:150, and added to this plate. The detection reaction employed an anti-human IgG-peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA) and o-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich). The reaction was stopped with 3 M H_2SO_4, and the absorbance measured at 492 nm using a microplate reader (Reader ASYS Microplates v.1.4; Biochrom, Cambridge, Austria). All samples were analyzed in duplicate. Samples with an average absorbance greater than 0.68 were considered to be positive (Yoneyama, 2007). The assay reactivity was confirmed by against the positive and negative controls provided in each plate.

DNA Extraction

The obtained buffy coat samples were washed with phosphate buffered saline (10 mM sodium PBS, 0.15 M NaCl, pH 7.2) and centrifuged at 3,500 × *g* for 15 minutes. DNA was extracted by the guanidine-phenol method (Venazzi *et al.*, 2007), resuspended in 50 µL TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0), and stored at 4°C until further use. One positive control (10^4 *L. (V.) braziliensis* promastigotes in normal human blood) and one negative control (normal human blood) was included for each group of samples extracted.

Polymerase Chain Reaction

The primers *MP3H* (5′-GAA CGG GGT TTC TGT ATG C-3′) and *MP1L* (5′-TAC TCC CCG ACA TGC CTC TG-3′) (Lopez *et al.*, 1993) were used to amplify a 70 bp fragment of the minicircle kinetoplast (kDNA) of sub-gender *Leishmania (Viannia)*. The samples were amplified and subsequently subjected to agarose gel electrophoresis according to the protocol provided by Nietzke-Abreu *et al.* (2013). The reaction mixture (25 µL) contained 1 µM of each primer (Invitrogen), 1.5 mM $MgCl_2$, 1X enzyme buffer, 0.2 mM dNTP (Invitrogen), 1 U Taq DNA polymerase (Invitrogen), and 2 µL of the sample DNA. Amplification was performed in a thermocycler (Biometra, Gottingen, Germany). The PCR conditions for primers *MP3H/MP1L* were set as follows: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation (95°C; 1.5 minutes), annealing (55°C; 1.5 minutes), and extension (72°C; 2 minutes), and a final extension at 72°C for 10 minutes. The reaction products were stored at 4°C until further analyses. Ten microliters of the amplification products were analyzed by electrophoresis, on a 3% agarose gel stained with 0.1 µg/mL ethidium bromide, at a voltage of 10–15 V/cm. A positive control (1 pg *L. braziliensis* DNA) and a negative control (water) was added to each group of samples. DNA bands were visualized using a UV transilluminator (Macro Vue UV-20; Hoefer Inc., Holliston, MA, USA).

Indirect Immunofluorescence

Promastigote forms of *L. braziliensis* (MHOM/BR/1987/M11272) and an anti-human IgG-fluorescein isothiocyanate (FITC) conjugate (BioMérieux, Craponne, France) were employed for the detection of anti-*Leishmania* antibodies. Serum samples were serially diluted (starting from a 1/20 dilution) and

titers ≥ 40 were considered to be positive (Silveira *et al.*, 1999). An Imunocruzi antigen (Biolab, Rio de Janeiro, Brasil) and an anti-human IgG-FITC conjugate (BioMérieux) was used in the detection of anti-*Trypanosoma cruzi* antibodies. Serum samples were serially diluted from a starting dilution of 1/20, and titers ≥ 40 were considered to be positive (Silveira *et al.*, 1999).

Statistical Analysis

The obtained data was introduced to a Microsoft Excel® 2010 worksheet. The Stata 9.1® (Stata Corporation, College Station, TX, USA) program was employed for data analyses. The data was analyzed by the Chi-squared test and Student's *t*-test for independent samples, with a significance level of 5%.

Ethical Considerations

All study participants were provided with detailed information regarding the details of the study; the patients who agreed to participate in this study were asked to sign an informed consent form. The study was approved by the Permanent Committee on Ethics in Human Research of the Maringá State University, according to the report n. 153/2009.

Results

One hundred and fifty nine patients, residing in six municipalities in northwestern Paraná, Brazil, were included in this study. Of these, 104 were male and 55 were female subjects. The patients were aged between 18 and 88 years, with an average age of 45.8 ± 16.3, and a median age of 46 years.

Of the 159 samples analyzed, 19.5% (31) were determined to be positive for ACL by ELISA-IgG, with an absorbance ≥ 0.68 (Yoneyama, 2007). Twenty (64.5%) and 11 (35.4%) of the ELISA-positive samples were obtained from males and females, respectively. There was no statistically significant correlation between the gender and ELISA positivity (p = 0.973). The age of ELISA-positive individuals ranged between 18 and 88 years, with an average age of 46.1 ± 15.9 and a median age of 44 years; therefore, the age and ELISA positivity were not significantly correlated (p = 0.607).

The cities São Jorge do Ivaí (35.48%; 11) and Doutor Camargo (22.59%; 7) showed the highest number of subclinical cases (Table 1). No statistically significant differences were observed between the municipalities in terms of the distribution of ELISA-positive individuals (p = 0.447).

Of the 31 ELISA-positive samples identified, 5 were discovered to be IIF-positive for *Leishmania*, while 8 were IIF-positive for *T. cruzi*. All 159 individuals were PCR-negative for *Leishmania (Viannia)* (Figure 1).

Table 1. Distribution of 31 ACL-positive individuals, as determined by ELISA, according to the municipality of residence in the northwestern region of Paraná.

Municipality	Number of individuals analyzed	Number of ACL-positive individuals (%)
Cianorte	22	2 (6,45)
Doutor Camargo	29	7 (22,59)
Jussara	17	5 (16,12)
Maringá	4	2 (6,45)
São Jorge do Ivaí	63	11 (35,48)
Terra Boa	24	4 (12,91)
Total	159	31 (100)

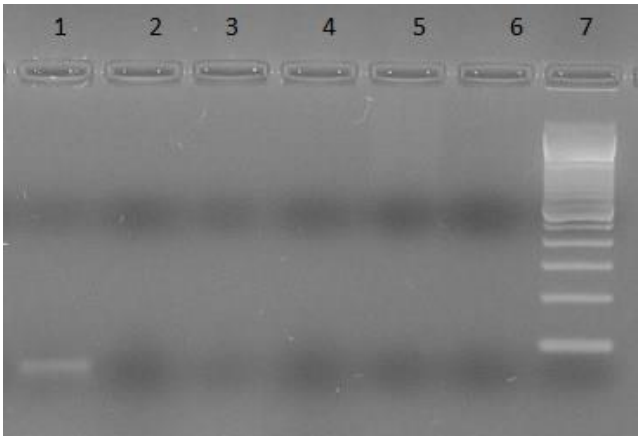

Fig. 1. Representative agarose gel showing PCR products of 70-bp (region of the minicircle kinetoplast kDNA of sub-gender Leishmania (Viannia), following amplification with MP3H and MP1L primers). Lane 1, positive control [DNA of L. (V.) *braziliensis* promastigote forms]; lane 2, negative control (water); lanes 3, 4, 5, and 6, DNA samples from study subjects; lane 7, MW, 100-bp molecular weight marker.

Discussion

The most common clinical manifestations of ACL are single, cutaneous ulcers, which correspond to localized lesions; these may evolve to healing and mucosal disease, which mainly affects the nasal and oral cavities, and whose treatment may be difficult (Brasil, 2009; Curti, 2009). Despite this, individuals living in endemic regions with no patent ACL-related lesions or scars may test positive for MST and serologically, suggesting a recent or subclinical infection (Nunes *et al.*, 2006; Arraes *et. al.*, 2008; Sampaio *et al.*, 2009). This study examined

antibodies of the IgG class. Thus, it is believed that positive cases are subclinical.

A majority of the individuals evaluated in this study were males (104/159); therefore, higher disease positivity was observed in males than females; for this reason, no statistically significant difference was observed between genders, in terms of ELISA-positivity. However, previous studies have reported greater frequency of the clinical manifestations of this disease in males than females (Monteiro, 2009; Curti *et al.*, 2009; Murback *et al.*, 2011).

Our results regarding the age of the patients are not in agreement with those seen in a previous study aimed at identifying the subclinical cases of ACL in the Federal District, which reported an average incidence age of 21.16 ± 14.01, and a median age of 19 years. This difference arises from the inclusion of children in the previous study, contrary to this study (Sampaio *et al.*, 2009).

The municipalities with the highest number of identified subclinical cases of were São Jorge do Ivaí and Doutor Camargo. However, no statistically significant differences were observed between the municipalities in terms of the distribution of detected subclinical cases. Previous studies have shown that ACL is endemic to the municipalities included in this study (Curti *et al.*, 2009). Between 1986 and 2006, 412 cases of ACL were reported in Maringá, 121 in São Jorge do Ivaí, and 126 in Doutor Camargo, the three municipalities with the highest prevalence of ACL in northwestern Paraná. The high ACL endemicity may explain the presence of subclinical forms of the disease in these municipalities. Previous studies have also reported the presence of subclinical cases in municipalities with high ACL endemicity (Arraes *et al.*, 2008; Sampaio *et al.*, 2009).

A previous study identified 11 (8.5%) ELISA-positive samples from among 130 healthy individuals living in an endemic region in Maringá city, Paraná (Arraes *et al.*, 2008), which is in agreement with the results presented in this study (19.5%). Some studies identified higher percentages of subclinical cases in the metropolitan area of Recife and the Federal District (67% and 71.8%), respectively (Brito *et al.*, 2008; Sampaio *et al.*, 2009), which the authors attributed to peri-domicile transmission. This is in contrast to the ACL transmission characteristics observed in northwestern Paraná, where the occurrence of ACL has been linked to proximity to woods and rivers, as well as to rural and peri-urban sections of endemic areas (Curti *et al.*, 2009).

Several authors have described the detection of *Leishmania (Viannia)* DNA in peripheral blood as an appropriate tool for the diagnosis of ACL (Ferreira, 2006; Venazzi, 2007; Martin, 2010). However, parasite DNA was not detected in the blood of individuals analyzed in this study. An analysis of patients with a clinical history of ACL conducted by Ferreira *et al.* (2006) did not reveal the presence of parasite DNA; however, the results of the ELISA conducted by Ferreira *et al.* (2006) were in agreement with those obtained in this study. Conversely, Camera *et al.* (2006) successfully detected parasite DNA in samples obtained from individuals (with no prior history of ACL) living in endemic regions.

In this study, DNA was extracted from the buffy coat extracted from the patient blood samples, which may have increased the difficulty of parasite DNA detection. Parasite DNA is frequently detected in whole blood and mononuclear cell samples, rather than in buffy coat samples (Camera, 2006). In addition, the detection of parasites in blood samples is believed to be related to the amount of circulating parasites. Venazzi *et al.* (2012) identified parasite DNA solely in those patients in whom a direct examination of the lesion samples revealed a high number of amastigotes. The samples analyzed in this study originated from subclinical patients, who are likely to have low parasitemias, or may even show parasite clearance, rendering the detection of parasite DNA impossible.

The association of different serological tests is needed to increase the positivity of LTA in the laboratory diagnostics (Yoneyama, 2007, Silveira *et al.*, 1999). Thus, IIF-*Leishmania* was performed to compare the positivity of the ELISA-positive samples. The results of IIF were not in agreement with those obtained by ELISA, which may be a consequence of the higher sensitivity of the latter (Szargiki *et al.*, 2009). Some studies have shown that IIF-negative samples could test positive for ACL in ELISA (Ferreira, 2006), including the subclinical cases (Arraes *et al.*, 2008). Thus, the use of different antigens in sensitive techniques, such as ELISA, can provide more specific diagnosis.

In the routine, the IIF reaction is the most used for the diagnosis of ACL, but there are possibilities of cross-reactions, especially with Chagas disease, so, it was performed the IFI-*T. cruzi*. The number of individuals determined to be IIF-positive for *T. cruzi* was higher than those that were IIF-positive for *Leishmania*. However, all *T. cruzi* IIF-positive samples presented high absorbance values in the ELISA-*Leishmania* assay.

A major limitation of this study was the time span between ELISA and IIF, with the latter having been performed after the former. Although the samples were storage in ideal storage conditions (-30°C), antibody titers are known to decrease with time, which may lead to a higher number of negative results (Souza *et al.*, 2012).

Conclusion

This study demonstrates the occurrence of subclinical infections in the inhabitants of northwestern Paraná. Based on the complexity of the immunological events that take place throughout the clinical development of ACL, further immune-genetic studies of subclinical cases may help elucidate why some individuals present

clinical manifestations of the disease, while others remain resistant to ACL.

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Author's contributions

RSRC: sample collection, laboratory tests, manuscript preparation; TRN: laboratory tests, manuscript preparation; LSB: laboratory tests; EPL: laboratory tests; CMS: laboratory tests; CCC: laboratory tests; SDB: manuscript preparation and revision; TGV: laboratory tests, manuscript preparation and revision.

All authors have read and approved the submitted version of the manuscript.

Competing interest

The authors have no conflicts of interest to declare.

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CAPÍTULO III

CONCLUSÕES

Neste trabalho detectamos casos subclínicos de LTA, entre indivíduos saudáveis moradores de áreas endêmicas, e demonstramos o grupo alélico HLA-C*04 como um possível marcador na susceptibilidade à LTA.

PERSPECTIVAS FUTURAS

O avanço da imunogenética tem permitido melhor entendimento da associação do HLA com várias doenças, contribuindo para a elucidação da patogenia, diagnóstico e prognóstico dessas enfermidades. Os resultados encontrados apontam a associação HLA de classe I e LTA. Estão sendo conduzidos estudos de associação com as moléculas de classe II (-DRB1, -DQA1 e –DQB1) e seus haplótipos.

Devido à alta miscigenação da população brasileira, resultados de estudos realizados em uma única região não devem ser generalizado para todo o país. As populações de outras regiões devem ser pesquisadas e estes e outros marcadores genéticos analisados para que novas associações possam ser identificadas ou confirmadas com outros estudos já publicados. Tal conhecimento contribuirá para prognósticos e intervenções terapêuticas em grupos brasileiros com maior risco de desenvolver a LTA.

Diante da complexidade dos eventos imunológicos envolvidos no curso clínico da LTA, acreditamos que os marcadores genéticos poderão contribuir para estabelecer o envolvimento de genes na susceptibilidade/resistência à LTA. E ainda, tentar esclarecer os mecanismos imunológicos envolvidos nos casos subclínicos.