Ministério da Saúde
Fundação Oswaldo Cruz
Centro de Pesquisas René Rachou
Programa de Pós-graduação em Ciências da Saúde

ABORDAGEM CLÍNICA DA LEISHMANIOSE VISCERAL ENTRE ADULTOS INFECTADOS PELO HIV: ACURÁCIA DIAGNÓSTICA, FATORES PROGNÓSTICOS E EFICÁCIA TERAPÊUTICA

por

GLÁUCIA FERNANDES COTA

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GLÁUCIA FERNANDES COTA

Tese apresentada com vistas à obtenção do Título de Doutor em Ciências na área de concentração Doenças Infecciosas e Parasitárias

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“Não, meu coração não é maior que o mundo.
É muito menor. Nele não cabem nem as minhas dores.
....
Sim, meu coração é muito pequeno.
Só agora vejo que nele não cabem os homens.
Os homens estão cá fora, estão na rua.
A rua é enorme. Maior, muito maior que eu esperava.
Mas também a rua não cabem todos os homens.
A rua é menor que o mundo.
O mundo é grande.
Tu sabes como é o mundo.
Vistes as diferentes cores dos homens
as diferentes dores dos homens
sabes como é difícil sofrer tudo isso, amontoar tudo isso, num só peito de homem....
sem que ele estale.
Entretanto alguns trouxeram a notícia
de que o mundo, o grande mundo
está crescendo todos os dias
entre o fogo e o amor,
entre a vida e o fogo,
meu coração cresce dez metros e explode
- ó vida futura! nós te criaremos.”

Carlos Drummond de Andrade
Sentimento do Mundo

APRESENTAÇÃO

Esta tese é composta por três revisões sistemáticas e um estudo clínico e busca contribuir para a redução de algumas lacunas do conhecimento acerca da coinfecção *Leishmania*-HIV.

Considerando a revisão da literatura uma investigação científica e a primeira fase de abordagem de um problema, esse trabalho se inicia com a busca dos fatores relacionados à recorrência da leishmaniose visceral (LV) entre infectados pelo vírus HIV no artigo "***Predictors of Visceral Leishmaniasis Relapse in HIV-Infected Patients: A Systematic Review****".* Tem continuidade com o artigo **"*The Diagnostic Accuracy of Serologic and Molecular Methods for Detecting Visceral Leishmaniasis in HIV Infected Patients: Meta-Analysis****",* que descreve a acurácia dos métodos sorológicos e moleculares para o diagnóstico da forma visceral da leishmaniose em pacientes portadores de HIV e, finalmente, uma revisão sobre os dados disponíveis a cerca da eficácia dos vários tratamentos utilizados para LV entre infectados pelo HIV no artigo ***"Efficacy of anti-Leishmania therapy in visceral leishmaniasis among HIV infected patients: a systematic review with indirect comparison".*** Em consonância com as regras do Programa de Pós-graduação em Ciências da Saúde do Centro de Pesquisa René Rachou, os artigos foram redigidos de acordo com as normas da revista ***PLoS Neglected Tropical Diseases,*** periódico médico eletrônico e de livre acesso onde estão publicadas as revisões. O quarto artigo, publicado no ***American Journal of Tropical Medicine and Hygiene*** e intitulado ***"Comparison of parasitological, serological and molecular tests for visceral leishmaniasis in HIV-infected patients: a cross-sectional delayed type study",*** relata a experiência de um centro de referência para doenças infecciosas em Minas Gerais com o diagnóstico de LV entre infectados pelo HIV e representa os primeiros resultados de um estudo de coorte iniciado em 2011.

SUMÁRIO

LISTA DE FIGURAS

FIGURAS DO ARTIGO 1

FIGURAS DO ARTIGO 2

FIGURAS DO ARTIGO 3

FIGURAS DO ARTIGO 4

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

ARTIGO 4

LISTA DE ABREVIATURAS E SÍMBOLOS

BLOT: do inglês, immunoblottin
CPqRR: Centro de Pesquisas Clínicas René Rachou
DAT: do inglês, direct aglutination test
DOR: do ingles, diagnostic odds ratiio
ELISA: do inglês, enzyme-linked immunosorbent assay
FDA: do inglês, Food and Drug Administration
FIOCRUZ: Fundação Oswaldo Cruz
FHEMIG: Fundação Hospitalar do Estado de Minas Gerais
FUNED: Fundação Ezequiel Dias
HEM: Hospital Eduardo de Menezes
HIV: do inglês, human immunodeficiency vírus
IFAT: do inglês, indirect immunofluorescent test
LPC: Laboratório de Pesquisas Clínicas
LT: leishmaniose tegumentar
LV: leishmaniose visceral
mL: mililitros
MO: medula óssea
OMS: Organização Mundial de Saúde
PCR: do inglês, polymerase chain reaction
qPCR: PCR em tempo real
ROC = do inglês, receiver operator characteristic
rK39: do inglês, recombinant antigen k39
SINAN: Sistema de Informação de Agravos de Notificação
SICLOM: Sistema de Informações sobre Mortalidade, Laboratório e Medicamento
sROC: summary ROC
SSrRNA: do inglês, small subunit ribosomal RNA
STARD: do inglês, Standards for Reporting of Diagnostic Accuracy
TARV: terapia antirretroviral
T CD4+: linfócito T CD4
VS-PBH: Vigilância em Saúde da Prefeitura de Belo Horizonte
LCM: do inglês, latente class model

RESUMO

Leishmaniose visceral (LV) entre infectados pelo HIV tem incidência crescente em regiões onde as duas infecções são endêmicas. Reconhece-se hoje que a doença está associada a maior mortalidade, menor taxa de resposta clínica e parasitológica e maior toxicidade ao tratamento que as observadas em pacientes não infectados pelo HIV. Várias são as incertezas e dificuldades a cerca do diagnóstico, tratamento e seguimento dos pacientes coinfectados com Leishmania-HIV. Esta tese é composta por três revisões sistemáticas e um estudo clínico e busca contribuir para a redução de algumas lacunas do conhecimento à cerca da coinfecção Leishmania-HIV. Por meio de revisões sistemáticas da literatura e de um estudo transversal, que comparou a acurácia de métodos invasivos e não invasivos para o diagnóstico de LV entre infectados pelo HIV, confirmamos baixa sensibilidade dos testes sorológicos, à exceção do teste de aglutinação direta, que deve ser preferido em rotinas de investigação. Incluindo nossos próprios resultados, foram identificados apenas três estudos que avaliaram o desempenho de testes imunocromatográficos rápidos, baseados na pesquisa do anticorpo contra o antígeno recombinante K39, entre infectados pelo HIV. Por sua vez, testes baseados na reação em cadeia da polimerase, incluindo a técnica que utiliza como alvo a subunidade ribossomal do RNA e testada em nosso meio, apresentam alto desempenho global e despontam como alternativa menos invasiva ao exame parasitológico. De acordo com a evidência disponível, a terapia antirretroviral altamente potente não constitui intervenção suficiente para evitar a recidiva. Por outro lado, o uso de profilaxia secundária reduz significativamente a ocorrência de episódios subsequentes de LV. A revisão da literatura nos permitiu ainda identificar algumas condições marcadoras do risco de recidiva que, se presentes, reforçam a indicação de profilaxia secundária: ausência de elevação da contagem de linfócitos T CD4+ no seguimento; história prévia e recidiva de LV; contagem de linfócitos T CD4+ abaixo de 100 células/mL na ocasião do primeiro diagnóstico de LV. Os trabalhos publicados revelam maior taxa de resposta clínica com o uso de anfotericina B em relação ao tratamento com derivados de antimônio, o que parece estar relacionado à menor toxicidade que à maior eficácia. Os derivados de antimônio são drogas mal toleradas pelos pacientes coinfectados com HIV, associando-se a alta taxa de descontinuidade do tratamento e mortalidade três vezes maior que a observada com o tratamento com anfotericina B. Os dados disponíveis até o momento são

insuficientes para se comparar a eficácia entre as várias formulações de anfotericina ou se definir a dose e o tempo de tratamento ideais para LV entre infectados pelo HIV.

ABSTRACT

Concurrent visceral leishmaniasis (VL) and HIV infection have been reported in most areas of the world where the geographical distribution of the two infections overlap. The disease is characterized by significantly lower cure rates, higher drug toxicity, relapse and mortality rates than those for VL in non-HIV-infected individuals. There are many uncertainties and difficulties about the diagnosis, treatment and monitoring of Leishmania-HIV coinfected patients. This dissertation is composed of three systematic reviews and a clinical study and aims to contribute to the reduction of some of the knowledge gaps in Leishmania-HIV coinfection field. Through a systematic literature review and a cross-sectional study, designed to evaluate the accuracy of invasive and noninvasive tests for VL diagnosis in HIV-infected patients, it was confirmed the low sensitivity of serological tests, except for direct agglutination test. Including our own results, there are only three studies evaluating the performance of anti-rK39 based dipsticks tests among HIV-infected patients. Tests based on DNA detection are highly sensitive and may contribute to a VL diagnostic workup. A good performance was also obtained (in our cross-sectional study) with a real time PCR (polymerase chain reaction) using as target the small subunit of ribosomal RNA. PCR tests are emerging as a less invasive and a useful alternative to parasitological examination. According to the available evidence, highly active antiretroviral therapy is not sufficient to prevent VL recurrence. In contrast, secondary prophylaxis was shown to be protective against relapse. Some predictors of VL relapse could be identified: a) the absence of an increase in CD4+ cells at follow-up; b) lack of secondary prophylaxis; and c) previous history of VL relapse. CD4+ counts below 100 cells/mL at the time of primary VL diagnosis may also be a predictive factor for VL relapse. Based on these observations, a high-risk population might be identified and such patients might then be eligible for secondary prophylaxis. Available evidence suggests higher clinical response rate with amphotericin B than with antimony treatment, which appears to be related to less toxicity than with higher effectiveness. Antimonial therapy carries a higher rate of drug discontinuation and a significantly higher mortality indirectly compared to treatment with amphotericin B. The optimal dose of amphotericin and the difference in efficacy between its various formulations remain to be established.

1 INTRODUÇÃO

1.1 **Aspectos epidemiológicos**

A leishmaniose representa um complexo de doenças com importância clínica e epidemiológica que afeta principalmente indivíduos de países em desenvolvimento, sendo considerada pela Organização Mundial de Saúde (OMS) [1] como uma das doenças tropicais negligenciadas. São mais de 14 milhões de pessoas infectadas em todo o mundo e cerca de dois milhões de novos casos a cada ano, dos quais apenas 600 mil são oficialmente notificados [2]. A doença é causada por um protozoário do gênero *Leishmania spp.* que se multiplica em vertebrados específicos, os reservatórios, sendo o parasito transmitido ao homem através da picada de flebotomíneos. A infecção afeta as camadas mais pobres das populações de 88 países onde 350 milhões de pessoas vivem sob risco [1].

São reconhecidas duas grandes apresentações clínicas de leishmaniose: a forma tegumentar (LT), que inclui as formas cutânea, mucocutânea e mucosa, e a visceral (LV). A diferenciação para uma ou outra forma da doença decorre da espécie de *Leishmania* envolvida e da resposta imune do hospedeiro. A forma cutânea pode seguir para cura espontânea deixando cicatrizes e, dependendo da espécie de *Leishmania*, a infecção pode evoluir para a leishmaniose cutânea difusa, recidivante ou mucosa, com lesões muitas vezes desfigurantes.

Leishmaniose visceral, também conhecida como Kala-azar, a forma sistêmica e mais grave dentre as leishmanioses, é fatal em quase todos os casos se não tratada [3]. É causada por parasitos do complexo donovani, havendo poucos casos descritos por *L.(L.) tropica* [4] e *L.(L.) amazonensis* [5]. A presença de desnutrição e imunossupressão, nomeadamente a infecção pelo HIV, são condições que reconhecidamente predispõem à manifestação clínica. Os sintomas comuns são febre, calafrios, perda de peso, anorexia e desconforto no hipocôndrio esquerdo. Os sinais clínicos mais frequentes são esplenomegalia, com ou sem hepatomegalia, e palidez das mucosas. Linfadenopatia pode estar presente, o que é especialmente comum no Sudão, podendo ser a única manifestação clínica. Escurecimento da pele da face, mãos, pés e abdome são tipicamente encontrados na Índia (na língua Hindi, kala-azar, significa "Febre negra" ou "Febre mortal"). Sinais de desnutrição (edema,

alterações da pele e cabelo) podem desenvolver-se à medida que a doença progride e infecções bacterianas intercorrentes são comuns.

Sob o aspecto da transmissão, são reconhecidas duas formas de LV: zoonótica e antroponótica. A forma zoonótica, causada por *L. (L.) infantum* e que tem o cão como principal reservatório, ocorre na região do Mediterrâneo, China, Oriente Médio e América do Sul. Ao nível global, a forma antroponótica, caracterizada pela transmissão homem-a-homem e sem reservatório animal, é claramente a mais comum, sendo responsável por mais de dois terços dos casos de LV no mundo. É causada por *L.(L.) donovani* e prevalente na África Oriental, Bangladesh, Índia e Nepal, locais densamente povoados [4, 6].

Em áreas endêmicas, a doença tende a ser relativamente crônica e as crianças são especialmente afetadas. Até recentemente, a faixa etária mais acometida por *L. (L.) infantum* no sul da Europa, norte da África e Ásia ocidental e central estava compreendida entre um e quatro anos. No entanto, desde o advento da infecção pelo HIV e aumento do uso de imunossupressão para o transplante e quimioterapia, cerca de metade dos casos na Europa são agora descritos em adultos [7]. Em áreas endêmicas da África oriental e Índia, a maior incidência é encontrada em crianças e adultos jovens [4].

O Brasil figura como um dos seis países detentores de 90% dos casos globais de LV, com aproximadamente 80 mil casos de LV notificados e cerca de 4500 mortes de 1980 a 2011. Dados de notificação do Ministério da Saúde do Brasil revelam um aumento na incidência da doença, sendo que a média de casos registrados anualmente cresceu de 1.601 entre 1985 a 1989 para cerca de 3.800 no período compreendido entre 2005 e 2011 [8]. Além de incidência alta, o país vem sofrendo significativa modificação no comportamento epidemiológico da LV nos últimos 30 anos. A doença, historicamente reconhecida como uma endemia rural e de ocorrência focal, começou a invadir as grandes cidades brasileiras no início da década de 1980. Primeiramente foram atingidas as cidades de Teresina/PI e São Luís/MA e, a seguir, focos de LV foram registrados em diferentes cidades do país como Santarém/PA, Natal/RN, Corumbá/MS, Montes Claros/MG, Belo Horizonte/MG, Campo Grande/MS, Aracaju/SE, Feira de Santana/BA, Araçatuba/SP, Bauru/SP, Imperatriz/MA, Palmas/TO, Fortaleza/CE, entre outras [9]. Recentemente,

casos de LV vêm se multiplicando nas regiões Centro-Oeste, Norte e Sudeste. Na década de 1990, apenas 10% dos casos do país ocorriam fora da Região Nordeste em contraste com os 47,5% em 2011 [8]. Os motivos que levaram à urbanização da leishmaniose são ainda pouco conhecidos, mas as transformações ambientais associadas a movimentos migratórios e a ocupação urbana não planejada, as condições precárias de saneamento e habitação nas periferias destas cidades e a desnutrição são alguns dos muitos fatores implicados no fenômeno.

Outra tendência epidemiológica que preocupa no Brasil é a elevação gradativa na letalidade por LV, que passou de 3,2% em 2000 para 5,7% em 2009 e 6.7% em 2011 [8, 10]. Por razões ainda não esclarecidas, a região metropolitana de Belo Horizonte, Minas Gerais, apresenta um dos índices de letalidade mais altos do país, com média de 12,6% entre 2006 a 2010, segundo informações disponíveis [11].

1.2 **Aspectos epidemiológicos da coinfecção *Leishmania*-HIV**

Segundo dados da OMS, já eram cerca de 34 milhões de pessoas em todo o mundo vivendo com HIV em 2010 [4]. O Brasil é o epicentro da epidemia na América do Sul, concentrando 1/3 de todas as pessoas com HIV da parte sul do continente americano [12]. Desde o primeiro relato brasileiro de infecção pelo HIV na década de 1980, já foram notificados até agora ao Ministério da Saúde quase 650.000 casos [8]. De nota, observam-se modificações importantes no perfil da epidemia nos últimos anos: dispersão dos casos para as macrorregiões com taxas mais baixas de urbanização e para as cidades de médio e pequeno porte.

Sobre a coinfecção *Leishmania*-HIV, o primeiro paciente diagnosticado no mundo com a condição foi apresentado em 1985 na França [13] e, desde então, a associação já foi descrita em cerca de 35 países [12]. Embora o número de novos casos tenha diminuído na Europa desde o final dos anos 1990 [7], principalmente devido ao acesso à terapia antirretroviral (TARV) altamente ativa, em outras partes do mundo, com acesso limitado ao tratamento, a prevalência da coinfecção continua a aumentar, especialmente em algumas regiões na África, como o norte da Etiópia. Neste país, a taxa de infecção pelo HIV entre pacientes com LV subiu de 19% entre 1998 e 1999 para 34% entre 2006 e 2007. Já no Brasil, Índia, Nepal e Sudão, a

prevalência estimada de infecção pelo HIV entre pacientes com LV mantem-se abaixo de 10%, mas com expectativa de elevação diante da ruralização da epidemia pelo HIV [4]. Uma avaliação de 83 casos de coinfecção descritos no Brasil desde 1987 em congressos, artigos científicos e notificações à rede de coinfecção *Leishmania*-HIV [14] revelou que 63% deles apresentavam LT (80% destes com a forma cutânea mucosa ou mucosa) e 37% LV. A média de idade foi de 37 anos e 91,9% dos casos eram do sexo masculino. Em 18% dos pacientes o diagnóstico de leishmaniose antecedeu o de HIV e, em 41%, o diagnóstico das duas infecções foi simultâneo.

Estudos recentes, realizados em população de pessoas infectadas pelo HIV no Brasil [15, 16], identificaram prevalência de infecção assintomática por *Leishmania spp.* entre 15 e 20%, avaliada por testes sorológicos ou moleculares, o que reforça a importância da coinfecção em nosso meio. Já sobre os indivíduos doentes, as maiores informações sobre a associação *Leishmania*-HIV no Brasil provêm do levantamento realizado, em 2006, pelo Ministério da Saúde a partir do cruzamento de dados dos Sistema de Informação de Agravos de Notificação (SINAN) e do Sistema de Informações sobre Mortalidade, Laboratório e Medicamento (SICLOM). Foram relacionados 16.210 casos de LV e 150 mil casos de LT que, após cruzados com o banco de notificações de aids, permitiram a identificação de 176 pacientes com LV/aids (1,1% dos casos de LV) e 150 casos com LT/aids (0,1% dos casos de LT). As principais manifestações clínicas da LV foram: febre (78%), emagrecimento (77%), esplenomegalia (91%) e hepatomegalia (55%). Infecções oportunistas concomitantes foram descritas com as seguintes frequências: candidíase (46%), pneumonia por *Pneumocystis jiroveci* (24%), infecção por *Mycobacterium* sp. (33%), neurotoxoplasmose (15%) e meningite criptocóccica (7%). Foram estes dados que demonstraram a magnitude da associação leishmaniose-aids no Brasil e apontaram para a necessidade de aprimoramento da vigilância da coinfecção.

Ao contrário do que se constata com a apresentação da doença, muito parecida com a observada em indivíduos sem imunodeficiência, a evolução clínica após o tratamento de LV em infectados pelo HIV diverge significativamente em relação ao curso da doença em imunocompetentes [2]. LV-HIV caracteriza-se por baixas taxas de cura [17, 18, 19], alta mortalidade [18, 19, 20], alta ocorrência de toxicidade durante o tratamento [18, 21] e, principalmente, grande tendência à recidiva [18, 20,

21, 22].

A taxa de recidiva de LV, os fatores relacionados à sua ocorrência, o impacto da elevação da contagem de linfócitos T CD4+ e da introdução de TARV altamente potente são desconhecidos e foram motivação para a primeira revisão da literatura, apresentada como **ARTIGO 1.** O conceito atual é de que LV ocorrendo no curso da infecção pelo HIV não pode ser curada em grande parte dos pacientes, apesar de extensivo tratamento leishmanicida [2]. Recentemente, a partir das informações obtidas com o acompanhamento, por meio de biologia molecular, de pacientes coinfectados pelo HIV após o tratamento para LV foi proposta a definição de uma nova entidade clínica denominada "leishmaniose visceral crônica ativa" [23]. A principal observação do grupo francês que primeiro propôs esta condição foi a ocorrência de episódios agudos de LV seguidos por períodos livres de sintomas, ou com sinais subclínicos, mas com persistência parasitária medida por PCR (do inglês, *polymerase chain reaction)*. Outra característica comum a esses casos é a falha parasitológica ao fim de vários tratamentos e a despeito do uso de profilaxia secundária, além de uma reconstituição imune débil, mesmo com o uso regular de TARV. Vários são os mecanismos aventados para explicar esse fenômeno, tal como a produção insuficiente de interleucina-2 e interferon gama, mediadores essenciais da resposta inflamatória, o que explicaria a circulação crônica de parasitos. Outra hipótese complementar é a existência de "santuários" que funcionariam protegendo o parasita da ação do sistema imune e das concentrações elevadas de anfotericina B.

Os sinais e sintomas clássicos de LV, febre, esplenomegalia e citopenia, isoladamente ou em combinação, não são específicos o suficiente para diferenciar esta condição de esquistossomose (aguda ou hepatoesplênica), salmonelose, histoplasmose, tuberculose disseminada, malária e outras infecções sistêmicas. Para pacientes infectados pelo HIV, o diagnóstico pode ser ainda mais difícil já que as manifestações típicas podem estar ausentes. De acordo com algumas séries publicadas, esplenomegalia é menos frequente entre coinfectados [18] e 42 a 68% dos casos apresentam outras infecções oportunistas associadas, que podem cursar com sintomas semelhantes, complicando ainda mais o diagnóstico clínico [24]. Nos últimos anos, além da pesquisa direta, cultivo e dos testes sorológicos, várias técnicas moleculares foram desenvolvidas, com grande avanço na qualidade de

identificação e caracterização de espécies de *Leishmania*, mas falta padronização e principalmente acesso a essas técnicas.

1.3 **Considerações sobre o diagnóstico de LV entre infectados pelo HIV**

1.3.1 **Diagnóstico parasitológico**

A detecção de parasitos por microscopia ou cultura em amostras de diferentes tecidos é considerada o "padrão ouro" ou "teste de referência" para o diagnóstico de LV entre doentes coinfectados com HIV. O aspirado esplênico, procedimento comum em regiões endêmicas na África [17, 25], é considerado o método mais sensível para o diagnóstico de LV, envolvendo porém risco não desprezível de complicação hemorrágica (aproximadamente 0,1%) [26]. O potencial de complicação dessa técnica exige sua realização por equipe experiente e em local com infraestrutura mínima para a abordagem de intercorrências. Fora da África, a pesquisa direta do parasito em material aspirado de medula óssea tem sido a técnica mais utilizada, com sensibilidade descrita entre 67 a 94% [18, 27-29]. Tão invasiva quanto o aspirado esplênico, a biópsia hepática tem sensibilidade descrita em 87% [29]. Por outro lado, a pesquisa de amastigotas em sangue periférico - uma alternativa diagnóstica minimamente invasiva - apresenta menor taxa de positividade, em torno de 50 a 53 % para pacientes coinfectados [30, 31], sendo que cultura de células mononucleares de sangue periférico tem sensibilidade entre 64 e 67% [32, 33]. Da mesma forma, o aspirado de linfonodos representa estratégia menos sensível para o diagnóstico, mas ainda frequentemente utilizado no Sudão, onde linfadenomegalia é um sinal comum [34, 35]. Muitas vezes como um achado ocasional e inesperado, amastigotas podem também ser encontradas em locais incomuns, tais como pulmões, laringe, amígdalas, aparelho digestivo, reto, fluido espinhal e outros [22]. Para o exame parasitológico, independentemente do espécime biológico utilizado, recomenda-se que o exame microscópico seja feito por ao menos uma hora com a observação de ao menos duas lâminas a procura das formas típicas do parasito. A experiência do microscopista é crucial para o diagnóstico preciso e, embora os imunossuprimidos sejam frequentemente descritos como maciçamente infectados, há relatos de resultados falsos negativos atribuídos à pancitopenia. A carga parasitária pode também ser afetada pelos tratamentos anteriores ou infecções

concomitantes [36]. Quanto ao desempenho da cultura, exame realizado com material obtido de aspirado esplênico mostra alta sensibilidade (63 a 100%) [18, 20], semelhante à encontrada com aspirado de medula óssea (50 a 100%) [22, 37].

1.3.2 **Diagnóstico sorológico**

Testes sorológicos são métodos não invasivos, simples, mas com valor diagnóstico reconhecido como limitado com base em relatos que estimam em mais de 40% a proporção de indivíduos coinfectados com HIV que não apresentam níveis mensuráveis de anticorpos específicos contra *Leishmania* spp. [22]. Esse padrão provavelmente decorre da ausência de células T que podem reconhecer antígenos de *Leishmania spp.,* o que é essencial para a apresentação e estimulação das células B. A taxa de soropositividade descrita na literatura varia de acordo com o teste sorológico empregado e difere significativamente entre os trabalhos publicados e nas diversas regiões endêmicas. Em uma revisão narrativa (não sistemática) de 2008 [2], a sensibilidade diagnóstica das técnicas sorológicas para pacientes coinfectados com HIV foi assim resumida: “entre 11 a 67% para imunofluorescência indireta (IFAT) [38, 39], 76 a 89% para teste imunoensaioenzimático (ELISA) usando antígeno solúvel [40], 22 a 62% para teste imunoensaioenzimático usando antígeno recombinante K39 (rK39) [38, 40], 74 a 85% para *immunoblotting* (BLOT) [38-40] e 95% para aglutinação direta (DAT) [41]”. O teste rápido utilizando antígeno rK39 é uma técnica sorológica de fácil execução e não invasiva, com alta sensibilidade e especificidade no diagnóstico de LV em pacientes imunocompetentes de diferentes países. Em relação a pacientes imunossuprimidos pela infecção pelo HIV, contudo, os dados são escassos e, tal como para as outras técnicas sorológicas, o desempenho descrito até o momento não atinge níveis satisfatórios [40]. Assim, apesar da existência de algumas publicações descrevendo medidas de acurácia para várias técnicas no diagnóstico de LV entre infectados pelo HIV, uma reunião sistematizada desta evidência ainda não havia sido feita, justificativa para a segunda revisão apresentada no **ARTIGO 2.**

1.3.3 **Detecção do antígeno de *Leishmania spp.***

Em teoria, testes baseados na detecção do antígeno deveriam ser mais sensíveis, por prescindirem da resposta imune, e mais específicos do que a pesquisa do anticorpo, por evitarem a reatividade cruzada e poderem distinguir infecção ativa de infecções passadas [42]. Baseado neste princípio desenvolveu-se um teste de aglutinação (KATEX®; Kalon Biological LTDA, Reino Unido) para detectar antígenos de *Leishmania* spp. em urina, durante episódios de doença ativa, quando a carga parasitária se encontra elevada. Em estudos realizados em pacientes coinfectados na Espanha [43, 44], a sensibilidade variou de 85,7% e 100% e a especificidade testada em único estudo foi de 96%. Riera e colaboradores [43] descreveram ainda que a pesquisa do antígeno foi negativa para pacientes coinfectados considerados curados (sem amastigotas em aspirado de medula óssea ou sangue após o tratamento), indicando que esse teste poderia ser utilizado como marcador de infecção ativa e monitoramento da eficácia do tratamento. Por outro lado, o teste detectou também antígenos de *Leishmania spp.* em períodos assintomáticos entre recaídas, indicando a presença de infecção subclínica. Estes resultados estão de acordo com aqueles obtidos em imunocompetentes pelos grupos liderados pelos pesquisadores Sundar [45] na Índia, El-Safi [46] no Sudão e por Cruz [47] na Espanha – este com pacientes pediátricos. Em outro estudo espanhol [48], a pesquisa do antígeno de *Leishmania* spp. foi negativa na urina de paciente infectado pelo HIV, assintomático, mas com infecção documentada por PCR, o que levanta mais questões sobre a aplicação do teste. Vários estudos realizados no leste da África e no subcontinente indiano [45, 49, 50] mostraram boa especificidade, mas uma sensibilidade de baixa a moderada (48-87%). Além desta limitação, duas outras de ordem práticas precisam ser ressaltadas: a urina deve ser fervida para evitar reações falso positivas e é difícil distinguir os resultados fracamente positivos dos negativos, o que afeta a reprodutibilidade do teste.

1.3.4 **Diagnóstico molecular**

A técnica de PCR para o diagnóstico das leishmanioses foi introduzida na década de 1990 [51, 52], inicialmente para a forma cutânea e em seguida para o diagnóstico da forma visceral, utilizando-se tecidos de pacientes infectados. Sua aplicação no

sangue foi primeiro descrita em 1995 [33, 53-55] e, desde então, foram muitas as publicações [56] em que o método, utilizando diferentes amostras clínicas, apesar da variação de resultados, alcançou desempenho melhor do que o obtido com os métodos parasitológicos clássicos [33, 57, 58]. Vários são os alvos utilizados, como RNA ribossômico (rRNA), DNA do cinetoplasto (kDNA) ou sequências nucleares repetidas [57, 59, 60]. O bom desempenho da técnica motivou seu uso frequente para o diagnóstico de LV em laboratórios de pesquisa e referência em muitos países, mesmo que usando protocolos artesanalmente desenvolvidos [61]. Por outro lado, a demonstração frequente de PCR positiva em indivíduos sem sintomas da doença em regiões endêmicas [62, 63], incluindo coinfectados pelo HIV [15, 16], lança alguma dúvida sobre sua aplicação clínica neste contexto. Mais recentemente, a PCR em tempo real (qPCR) permitiu ainda a quantificação da carga parasitária de maneira fidedigna e rápida, o que faz vislumbrar a possibilidade de mais uma ferramenta para determinação de infecção utilizando sangue periférico. Por esta técnica, o monitoramento da amplificação de sequências específicas de DNA ocorre à medida que a reação procede. Suas principais vantagens incluem a redução no tempo necessário para a realização do ensaio e a redução da taxa de exames positivos por contaminação [53, 64]. Especificamente no caso de doentes coinfectados com HIV, uma de suas aplicações mais relevantes seria o acompanhamento da dinâmica da infecção por *Leishmania spp.* após o tratamento e, possivelmente, a predição de recaídas em longo prazo [56, 60, 65]. Em cães doentes, já se evidenciou que cargas parasitárias mais altas, mensuradas através do qPCR, estão relacionadas a quadro clínico mais grave [66, 67]. Contudo, a relação entre maiores quantidades de parasitos e evolução para doença em cães infectados inicialmente assintomáticos é ainda controversa [68, 69]. Em humanos, os estudos com qPCR são escassos, havendo grande variabilidade na carga parasitária descrita e sua relação com a evolução clínica ainda não foi estabelecida [70]. Por outro lado, significativa redução da parasitemia logo após início do tratamento específico para LV é uma constante entre os trabalhos [53, 71, 72]. Em pacientes coinfectados pelo HIV, tal como em imunocompetentes, também se observa queda significativa no número de parasitos logo após início de administração de anfotericina B, havendo, contudo, persistência de carga parasitária baixa ao término do tratamento em grande parte dos pacientes e elevação da parasitemia concomitante à recidiva de LV [57, 56, 73].

1.4 **Tratamento de LV entre infectados pelo HIV**

Os compostos à base de antimônio (estibugluconato de sódio e antimoniato de meglumina) foram usados ao longo das últimas décadas como o fármaco de primeira linha para o tratamento de LV, inclusive em infectados pelo HIV, em virtude do seu baixo custo e disponibilidade na maioria dos países [2]. No entanto, são drogas bem conhecidas por sua alta toxicidade e resistência emergente. Em relação aos portadores de HIV, são muitas as publicações sugerindo que a alta mortalidade observada no curso do tratamento para LV esteja relacionada ao próprio antimoniato [18, 19]. Desde a década de 80, a anfotericina B tem sido utilizada no tratamento de LV. Trata-se de um antifúngico poliênico cujo alvo é o ergosterol, principal componente da membrana da *Leishmania spp..* Seu uso também tem sido limitado por toxicidade e efeitos adversos, especialmente febre, calafrios, flebite e, principalmente, nefrotoxicidade. Em função destes efeitos adversos, recentemente, a anfotericina desoxicolato vem sendo substituída, em países com recursos financeiros suficientes, por uma de suas formulações lipídicas. Nos Estados Unidos da América, a formulação lipossomal (AmBisome®) é hoje a única droga aprovado pela *Food and Drug Administration* (FDA) para o tratamento de LV [74]. Estudos na Europa, Índia e Brasil demonstraram que a formulação lipossomal da anfotericina B tem alta eficácia e baixa toxicidade em pacientes imunocompetentes com VL [75]. Na África [76], contudo, já foi observado percentual significativo de falência terapêutica com a dose habitual de anfotericina lipossomal (20mg/kg), o que se especula estar relacionado com a presença de imunossupressão pelo HIV (não identificada) e cargas parasitárias mais altas para *Leishmania* spp. A pesar desta observação e de haver poucos trabalhos comparando os vários tipos de tratamentos na coinfecção *Leishmania*-HIV, anfotericina lipossomal vem sendo considerada o tratamento de escolha para este grupo de imunossuprimidos, baseado principalmente na opinião de especialistas [2, 74]. Na década passada, duas novas drogas foram adicionadas ao arsenal terapêutico para a leishmaniose: paromomicina e miltefosina. Esta última, uma droga originalmente desenvolvida como antineoplásico, constitui-se hoje na única opção disponível para administração oral. A primeira experiência com miltefosina em portadores de HIV foi relatada na África e indicou boa tolerância à droga, mas menor taxa de cura quando comparada com o tratamento com antimoniato [21]. Sobre a paromomicina, ainda não há dados sobre a sua aplicação em portadores do HIV. Por enquanto, tanto paromomicina quanto

miltefosina são drogas com disponibilidade limitada fora da Índia e alguns países da África. Ademais, nenhuma das duas drogas se encontra registrada para o tratamento de LV no Brasil. Outra possibilidade que já foi considerada há alguns anos um tratamento alternativo (segunda linha) para LV, isetionato de pentamidina, é hoje raramente utilizada devido à sua eficácia subóptima e alta toxicidade, particularmente diabetes mellitus insulinodependente irreversível. Diante da inexistência de uma droga ideal para o tratamento de LV, que reúna alta eficácia, baixa toxicidade e custo acessível, uma abordagem cada vez mais explorada é a terapia combinada, especialmente em regiões de alta endemicidade, com o objetivo de se identificar um esquema terapêutico de curta duração, barato, bem tolerado e que possa preferencialmente ser usado em regime ambulatorial, requerendo monitoramento clínico mínimo. A terapia combinada pode também ajudar a retardar o aparecimento de resistência e aumentar a vida útil das respectivas drogas, mesmo racional que tem sido utilizado para doenças como a malária, tuberculose e HIV. Várias combinações começam a ser avaliadas, inclusive com análises de custo efetividade [77, 78], tal como antimoniais com paromomicina, anfotericina lipossomal em dose única associada à paromomicina e/ou miltefosina [6], dentre outras. Até o momento, nenhum estudo fase 3 para avaliação de terapia combinada incluiu pacientes infectados pelo HIV. Outro ponto essencial a ser considerado na escolha terapêutica para uma doença de distribuição tão ampla são as diferenças na eficácia terapêutica entre as várias regiões endêmicas, o que é parcialmente explicado pelas diferenças de susceptibilidade entre as espécies de *Leishmania.* Coerente com essa observação, a OMS [79] recentemente preconizou diferentes tratamentos de acordo com a região geográfica. Sobre a terapia para coinfectados por *Leishmania*-HIV, entretanto, nenhuma recomendação formal baseada na compilação de evidências foi produzida. Com este fim foi realizada a revisão sistemática apresentada no **ARTIGO 3**, que pretendeu identificar os dados publicados sobre a efetividade e toxicidade relacionadas aos vários tratamentos descritos para LV em indivíduos infectados pelo HIV.

Ao compilar a evidência científica publicada sobre a abordagem clínica da coinfecção *Leishmania*-HIV torna-se evidente a escassez de informações à cerca da realidade da doença nas Américas e, em especial, no Brasil. Assim, com o objetivo de aumentar o conhecimento da coinfecção em nosso meio, o **ARTIGO 4**, que traz os primeiros resultados de uma coorte de pacientes com diagnóstico de LV iniciada

em 2011, apresenta uma avaliação comparativa do desempenho da palpação do baço, do exame parasitológico, das várias técnicas sorológicas e da PCR em tempo real - utilizando a subunidade ribossomal do RNA como alvo e realizada em sangue periférico, no diagnóstico de LV em um serviço de referência em Belo Horizonte, Minas Gerais.

2 OBJETIVOS

2.1 **OBJETIVO GERAL**

Estudar a abordagem clínica da leishmaniose visceral entre pacientes infectados pelo HIV em relação aos aspectos prognósticos, diagnósticos e terapêuticos.

2.2 **OBJETIVOS ESPECÍFICOS**

Revisar de forma sistematizada a evidência científica publicada sobre a forma visceral da leishmaniose em indivíduos infectados pelo HIV, em relação aos seguintes aspectos:

- incidência de recidiva e os fatores relacionados à sua ocorrência;
- acurácia dos métodos diagnósticos sorológicos e moleculares;
- eficácia e toxicidade relacionadas às opções terapêuticas.

Determinar e comparar em adultos infectados pelo HIV e residentes em uma área urbana, endêmica e com sintomas de LV, a acurácia de vários métodos diagnósticos, a saber: imunofluorescência indireta, aglutinação direta, teste rápido baseado na pesquisa do anticorpo contra o antígeno rK39, exame parasitológico de material obtido por aspiração de medula óssea e PCR em tempo real para *Leishmania (L.) infantum* no sangue periférico.

3 MÉTODOS

Para se alcançar os objetivos propostos foram realizados três revisões sistemáticas da literatura e um estudo transversal com seguimento prospectivo.

3.1 **METODOLOGIA DAS REVISÕES SISTEMÁTICAS DA LITERATURA**

A revisão sistemática constitui um dos instrumentos para obtenção de subsídios para prática baseada em evidência. É uma metodologia rigorosa proposta para identificar os estudos sobre um tema em questão e disponibilizar a sua síntese, aplicando métodos explícitos e sistematizados de busca. A metodologia busca avaliar a qualidade e validade desses estudos, além de sua aplicabilidade no contexto onde as mudanças serão implementadas. Cada um desses objetivos é planejado e registrado considerando critérios que os validam, para minimizar o viés e garantir qualidade à metodologia [80].

As revisões seguiram as recomendações da Colaboração Cochrane [80] e roteiro recentemente publicado [81], seguindo-se as etapas propostas pelo manual PRISMA [82]. De acordo com essas recomendações, o trabalho pôde ser dividido em:

1- Formulação da pergunta
2- Localização e seleção dos estudos
3- Avaliação da qualidade dos estudos
4- Coleta de dados
5- Análise e apresentação dos resultados
6- Interpretação dos resultados
7- Programação da atualização das revisões

3.1.1 **Bases de dados**

Foram realizadas buscas nas principais bases eletrônicas de acesso livre (PubMed e LILACS), além da base de dados de ensaios clínicos controlados da Colaboração Cochrane e busca manual nas referências bibliográficas dos artigos originais identificados. Para a revisão concluída em 2012, foram pesquisadas também as bases EMBASE e WEB OF KNOWLEDGE, que se tornaram recentemente disponíveis de forma gratuita para profissionais registrados no Conselho Federal de Medicina do Brasil. Para a busca na base de dados PubMed, foram utilizados descritores *MeSH* (*Medical Subject Headings)*, vocabulário em língua inglesa usado

para indexar artigos, combinados com palavras textuais [83] com o objetivo de aumentar a sensibilidade da busca.

3.1.2 **Critérios de inclusão**

Foram incluídos artigos originais, sem restrição de data de publicação ou idioma, que tivessem dados identificáveis de pacientes maiores de 14 anos infectados pelo HIV e com diagnóstico de LV definido pela presença de sinais e/ou sintomas associados à evidência parasitológica, sorológica ou molecular da infecção por *Leishmania spp.*

3.1.3 **Critérios de exclusão**

Para todas as revisões foram excluídos estudos com menos de 10 pacientes porque estudos com amostras pequenas apresentam maior chance de viés de publicação [84].

3.1.4 **Busca de artigos**

Os artigos foram extraídos independentemente, sempre por dois revisores, e os resultados das buscas foram comparados para verificar a concordância. Discordâncias foram resolvidas por consenso e, caso necessário, com base na opinião de um terceiro pesquisador experiente (AR).

3.1.5 **Extração de dados**

Elaborou-se em todos os casos um instrumento organizado para a extração dos dados, utilizando-se tabelas contendo as variáveis de interesse. Foram registradas as características metodológicas de cada estudo (desenho, tamanho e características da amostra, forma de diagnóstico ou seleção de pacientes, covariáveis e desfechos avaliados, tempo de seguimento, análise estatística), características dos pacientes estudados e, finalmente, as medidas dos desfechos de interesse para cada revisão.

3.1.6 **Avaliação da qualidade dos estudos**

A justificativa para a avaliação da qualidade dos estudos incluídos é a detecção das semelhanças (homogeneidades) e diferenças (heterogeneidades) entre eles, critérios fundamentais na decisão de agrupá-los para a realização de metanálise. O instrumento de avaliação de qualidade utilizado variou de acordo com o tipo de revisão, se prognóstica ou diagnóstica, e com o tipo de estudo avaliado, se randomizado ou não randomizado. Independente do instrumento aplicado, os aspectos analisados envolveram os métodos aplicados nos estudos originais, forma de seleção dos participantes, intervenção realizada e os desfechos avaliados. Na revisão sobre acurácia diagnóstica utilizou-se o método "Quality Assessment of Diagnostic Accuracy Studies" (QUADAS) [85], escore composto por 14 critérios e elaborado por um grupo designado pelo NHS R&D (*Network for those Involved in Managing and Supporting Research and Development in Health and Social Care) - Health Technology Assessment Programme (HTA)* do Reino Unido. Para a avaliação da qualidade dos estudos não randomizados, reunidos na revisão sobre eficácia terapêutica, utilizou-se o instrumento Newcastle-Ottawa Scale (NOS) [86], um sistema de classificação que pontua os estudos de acordo com três perspectivas: seleção dos participantes, comparabilidade entre grupos, forma de mensuração do desfecho.

3.1.7 **Abordagem da heterogeneidade**

Do ponto de vista metodológico, as fontes de heterogeneidade entre os estudos são muitas: o acaso, as diferenças de delineamento, a forma de seleção de pacientes, as diferenças nas intervenções terapêuticas aplicadas e na forma em que os exames foram avaliados. Outra causa de heterogeneidade importante e exclusiva dos estudos de exames diagnósticos e prognósticos é a variação nos pontos de cortes para os valores de referência do exame em questão. Mesmo em estudos randomizados para intervenção terapêutica, pode existir heterogeneidade porque a randomização não foi voltada para o exame em questão e sim para a intervenção terapêutica. Estudos retrospectivos por sua vez são enfraquecidos por causa de seu risco de viés de seleção. Já o viés de verificação ocorre, por exemplo, quando a indicação do exame padrão-ouro é influenciada pelo resultado do exame

investigado: por exemplo, se a probabilidade de ser submetido ao exame parasitológico em aspirado de medula (“padrão-ouro”) for maior naqueles com teste sorológico positivo do que naqueles com teste negativo. Também em relação ao viés de verificação, a análise do exame investigado deve ser idealmente mascarada para outros testes e para o desfecho. O viés causado pelo espectro de fases da doença provoca variações na sensibilidade e na especificidade do exame investigado, por comparar populações com fases diferentes de uma mesma doença: alguns estudos com a maioria dos pacientes numa fase leve e inicial e outros estudos com pacientes em fase avançada da doença [87]. Finalmente, o viés de publicação é a tendência de estudos com resultados positivos serem mais frequentemente publicados que estudos com resultados negativos, especialmente em revistas de maior impacto e em língua inglesa. Ocorre habitualmente porque tanto o autor como o editor apresentam resistência em publicar estudos com resultados negativos. Estudos com amostras muito pequenas apresentam maior chance de viés de publicação, motivo pelo qual alguns autores preconizam que sejam excluídos. Para reduzir a possibilidade de viés de publicação, as fontes de busca foram ampliadas ao máximo [88].

A heterogeneidade entre os diversos estudos foi avaliada por meio da estatística I^2, também chamada de medida de inconsistência. Essa estatística descreve a porcentagem de variabilidade do efeito que é devida à heterogeneidade e não ao acaso. Quando I^2 apresenta valor acima de 50%, considera-se que há heterogeneidade substancial, até 25% considera-se heterogeneidade leve e, e entre 25 e 50% , heterogeneidade moderada. Diante de heterogeneidade alta, a estratégica foi incorporar a heterogeneidade usando o modelo com efeitos aleatórios para a estimativa do efeito, o que aumenta o intervalo de confiança do efeito medido e produz uma medida mais conservadora ao permitir que a heterogeneidade interestudo seja considerada na medida final. Sempre que possível, tentou-se explorar a heterogeneidade identificando suas causas, seja por análise de subgrupos ou metarregressão [80].

3.1.8 **Descrição qualitativa dos resultados**

Na parte inicial dos resultados das revisões, foram feitas apresentações narrativas das principais características dos estudos selecionados e s uas populações.

3.1.9 **Descrição quantitativa dos resultados – metanálise**

A metanálise resultou da combinação dos resultados dos artigos originais, produzindo uma única medida do efeito da intervenção terapêutica, da acurácia do teste diagnóstico ou do f ator de risco em estudo. Combinando-se as evidências científicas pôde-se aumentar o tamanho da população analisada, reduzir o intervalo de confiança e aumentar o poder estatístico a fim de estimar com mais precisão o resultado final, ajustando a magnitude do seu valor e aum entando a força desta evidência [89]. Para cada estudo em cada revisão, foram observados desfechos clínicos dicotômicos para os quais se extraiu o número de eventos e o tamanho da amostra em cada grupo. Nos desfechos clínicos contínuos foram necessários, em cada grupo: média, desvio padrão e t amanho da amostra. O resultado final da metanálise resultou sempre em uma média ponderada das estimativas do efeito dos estudos.

3.2 **METODOLOGIA DO ESTUDO TRANSVERSAL**

3.2.1 **Desenho do estudo**

Trata-se de um corte transversal com seguimento clínico (*cross sectional delayed-type study*) em uma coorte de pacientes com suspeita de leishmaniose visceral, avaliados de 1º de março 2011 até 28 de fevereiro de 2013, no Hospital Eduardo de Menezes, uma unidade da F undação Hospitalar do Estado de Minas Gerais (HEM-FHEMIG) e c entro de referência em infectologia em Belo Horizonte, Minas Gerais. Do total de 178 pacientes com suspeita de LV que concordaram em participar do estudo, os resultados da investigação diagnóstica de 113 pacientes portadores de infecção pelo HIV constituem o ARTIGO 4 desta tese.

3.2.2 **Definição de caso suspeito de LV**

Pacientes com idade igual ou superior a 14 anos e ao menos um dos seguintes sinais ou sintomas:

a- febre há mais de 14 dias ou

b- citopenia ou

c- visceromegalia.

Definiu-se citopenia [91] como uma ou mais das seguintes condições:

a- contagem de leucócitos inferior a 3.500 células/mm^3

b- contagem de plaquetas inferior a 120.000 plaquetas/mm^3

c- hemoglobina inferior a 11,0g%

Definiu-se visceromegalia [92] como fígado palpável a mais de 2 cm do rebordo costal direito na linha hemiclavicular ou presença de baço palpável.

3.2.3 **Cálculo amostral**

Utilizando-se o software MedCalc® versão 9.4.2.0 estimou-se em 47 pacientes a amostra necessária para a comparação das áreas sob as curvas ROC (do inglês, *receiver operating characteristic*) produzidas para os resultados de DAT e IFAT, tomados como variável contínua e derivados dos mesmos casos. Esta estimativa considerou o nível de significância de 0,05 e poder de 0,80, partindo-se das estimativas de 0,78 e 0,92, baseadas em revisão da literatura [93], para as áreas sob a curva ROC para os testes IFAT e DAT, respectivamente. Estimou-se o coeficiente de correlação entre os exames no grupo positivo (doentes) e negativo (não doentes), em respectivamente 0,46 e 0,05; uma estimativa conservadora derivada de um estudo veterinário [94]. Mantendo-se todos os demais parâmetros e alterando-se os coeficientes de correlação nos grupos positivos e negativos para 0,81 e 0,05, respectivamente, com base em estudo em humanos [95], a amostra necessária seria de 49 pacientes. Outra comparação de interesse utilizada para o cálculo amostral baseou-se na correlação entre DAT e PCR. Da mesma forma que realizado anteriormente, considerando-se o nível de significância de 0,05 e poder de 0,80, e partindo-se das estimativas da área sob a curva ROC para DAT e PCR de respectivamente 0,92 e 0,98 (também com base na revisão da literatura [93]), seriam

necessários 99 pacientes, tendo como premissas coeficientes de correlação nos grupos positivos e negativos de 0,5 e 0,5 (baseando-se em estimativas conservadoras de Deborggraeve *et al*., 2008 [96]). Finalmente, considerou-se ainda que seria necessária amostra mínima de cem pacientes para a realização de análise utilizando modelo de classes latentes [97].

3.2.4 **Condução do estudo**

Os casos suspeitos de LV foram ativamente procurados por um dos pesquisadores através da avaliação sistemática de cada paciente internado no HEM-FHEMIG e, em caso de consentimento, submetidos à coleta de sangue periférico para realização de IFAT, teste rápido em soro baseado na pesquisa do anticorpo contra antígeno recombinante K39 (Kalazar Detect®), além de punção aspirativa de medula óssea (MO) para realização de pesquisa direta e cultura para *Leishmania spp*. As amostras para realização de IFAT foram encaminhadas à Fundação Ezequiel Dias (FUNED), conforme convênio instituído. O teste rápido e a pesquisa direta de amastigotas no esfregaço de MO foram realizados no laboratório do HEM-FHEMIG. Amostras de aspirado de MO para cultura foram encaminhadas ao Laboratório de Pesquisas Clínicas (LPC) do Centro de Pesquisas René Rachou (CPqRR). Importante ressaltar que, simultaneamente à propedêutica para LV, foram realizados os exames necessários para investigação de outras possibilidades diagnósticas, de acordo com o julgamento do médico assistente responsável pelo caso. O tratamento de pacientes diagnosticados como portadores de LV seguiu as recomendações do Ministério da Saúde do Brasil [9], que inclui anfotericina B desoxicolato, anfotericina lipossomal ou antimoniato de meglumina. Após o término do tratamento, segundo rotina do serviço, pacientes infectados pelo HIV com contagem de linfócitos T CD4 inferior a 350 células/mm^3 foram encaminhados para profilaxia secundária para LV com uso quinzenal de anfotericina B. Todos os pacientes com diagnóstico de LV foram convidados a se submeter a um acompanhamento clínico e laboratorial bimensal, incluindo dosagem de citocinas inflamatórias e parasitemia (*L. infantum)* através de qPCR, por 12 meses, após o término do tratamento.

4 RESULTADOS

ARTIGO 1

PLoS Negl Trop Dis 5(6): e1153. doi:10.1371/journal.pntd.0001153

"Predictors of Visceral Leishmaniasis Relapse in HIV-Infected Patients: A Systematic Review"

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PLoS NEGLECTED TROPICAL DISEASES

Predictors of Visceral Leishmaniasis Relapse in HIV-Infected Patients: A Systematic Review

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Abstract

Background and Objectives: Visceral leishmaniasis (VL) is a common complication in AIDS patients living in *Leishmania*-endemic areas. Although antiretroviral therapy has changed the clinical course of HIV infection and its associated illnesses, the prevention of VL relapses remains a challenge for the care of HIV and *Leishmania* co-infected patients. This work is a systematic review of previous studies that have described predictors of VL relapse in HIV-infected patients.

Review Methods: We searched the electronic databases of MEDLINE, LILACS, and the Cochrane Central Register of Controlled Trials. Studies were selected if they included HIV-infected individuals with a VL diagnosis and patient follow-up after the leishmaniasis treatment with an analysis of the clearly defined outcome of prediction of relapse.

Results: Eighteen out 178 studies satisfied the specified inclusion criteria. Most patients were males between 30 and 40 years of age, and HIV transmission was primarily via intravenous drug use. Previous VL episodes were identified as risk factors for relapse in 3 studies. Two studies found that baseline CD4+ T cell count above 100 cells/mL was associated with a decreased relapse rate. The observation of an increase in CD4+ T cells at patient follow-up was associated with protection from relapse in 5 of 7 studies. Meta-analysis of all studies assessing secondary prophylaxis showed significant reduction of VL relapse rate following prophylaxis. None of the five observational studies evaluating the impact of highly active antiretroviral therapy use found a reduction in the risk of VL relapse upon patient follow-up.

Conclusion: Some predictors of VL relapse could be identified: a) the absence of an increase in CD4+ cells at follow-up; b) lack of secondary prophylaxis; and c) previous history of VL relapse. CD4+ counts below 100 cells/mL at the time of primary VL diagnosis may also be a predictive factor for VL relapse.

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Introduction

Visceral leishmaniasis (VL) and human immunodeficiency virus (HIV) co-infection has emerged as a serious disease pattern [1,2]. HIV infection increases the risk of developing VL by 100 to 2,320 times in endemic areas [3,4] and, on the other hand, VL promotes the clinical progression of HIV disease and the development of AIDS-defining conditions [5]. Both infections switch the predominantly cellular immunological response from Th1 to Th2 through complex cytokine mediated mechanisms leading to a synergistic detrimental effect on the cellular immune response [6,7,8]. Other important findings related to HIV-*Leishmania* co-infection is a reduction in therapeutic response and high rate of relapse, which is the clinical deterioration after clinical improvement, observed in 25–61% of patients [9,10,11,12]. Although the term recurrence has also been used as synonym for relapse, recurrence applies to the finding of a parasite repeatedly. It is important to emphasize that neither of these two terms distinguishes parasitological persistence from re-infection.

The poor therapeutic outcome, the high rate of relapse, the poliparasitic nature of VL in HIV-infected persons, as well as the atypical manifestations of the disease and the impaired access to health-care resources make HIV-infected individuals prone to enlarge the number of human reservoirs [13]. This concern is of utmost importance in Asia, where HIV and *Leishmania* co-infections are increasingly being reported in countries that are also facing parasite resistance to antimonial drugs [14].

Recent changes in the epidemiological patterns of HIV and *Leishmania* infections are likely to lead to a greater degree of overlap and greater risk of co-infection and they justify increased alertness. From a global epidemiologic viewpoint, two parallel trends are alarming: the ruralization of the HIV pandemic and the urbanization and spread of VL [1,15]. World Health Organization (WHO) [16] reports that the public health impact of leishmaniasis worldwide has been grossly underestimated for many years because notification was compulsory in only 32 of the 88 countries where 350 million people were at risk. The reported global incidence of co-infection is likely underestimated either because

Author Summary

Visceral leishmaniasis (VL) is the most serious form of an insect-transmitted parasitic disease prevalent in 70 countries. The disease is caused by species of the *L. donovani* complex found in different geographical regions. These parasites have substantially different clinical, drug susceptibility and epidemiological characteristics. According to data from the World Health Organization, the areas where HIV-*Leishmania* co-infection is distributed are extensive. HIV infection increases the risk of developing VL, reduces the likelihood of a therapeutic response, and greatly increases the probability of relapse. A better understanding of the factors promoting relapses is essential; therefore we performed a systematic review of articles involving all articles assessing the predictors of VL relapse in HIV-infected individuals older than 14 years of age. Out of 178 relevant articles, 18 met the inclusion criteria and in total, data from 1017 patients were analyzed. We identified previous episodes of VL relapse, CD4+ lymphocyte count fewer than 100 cells/mL at VL diagnosis, and the absence of an increase in CD4+ counts at follow-up as major factors associated with VL relapse. Knowledge of relapse predictors can help to identify patients with different degrees of risk, facilitate and direct prophylaxis choices, and aid in patient counseling.

VL has not been included in the list of AIDS related opportunistic infection in all endemic areas.

Before the widespread use of antiretroviral therapy, such co-infection was common in Europe [5]. The co-infection is now becoming proportionately more prominent in areas with poor access to antiretrovirals, such as Africa. In areas where it is available, highly active antiretroviral therapy (HAART) has changed the course of the HIV/AIDS epidemic and the outcome of associated opportunistic infections. However, evidence of relapse rate reduction in patients using HAART is conflicting [17]. This work is a systematic review of studies describing the predictors of VL relapse in HIV-infected patients.

Methods

Search Strategy and Selection Criteria

This review was conducted on all papers published before July, 31, 2010. To ensure scientific rigour, the Preferred Reporting of Systematic Reviews and Meta-Analysis (PRISMA) guidelines [18] were used for systematic data synthesis. Studies were identified by a Medline/PubMed search using a combination of terms that has been shown to maximize sensitivity [19]. The search terms used are shown in Figure 1. The LILACS and Cochrane databases were used for literature review using a Boolean combination of search terms. Additional reports were located using a manual search of references from retrieved papers. Two independent reviewers (GFC and MRS) initially checked the lists of titles and abstracts identified by this search to determine whether an article contained relevant data. Studies were considered eligible if they were presented in an original article, examined HIV-infected individuals over 14 years of age with a VL diagnosis, included follow-up after the leishmaniasis treatment and clearly analyzed predictors of relapse.

There were no restrictions on the publication language, date of publication, use of secondary prophylaxis, or duration of follow-up in the study. We excluded studies evaluating fewer than ten cases and studies evaluating mixed populations of HIV-infected and uninfected subjects unless separated results for HIV patients were identified. The selected articles were read in full to confirm eligibility.

Data were extracted directly from the full-length articles into structured tables containing all of the descriptive variables and relevant outcomes. The following information was extracted: country and period of enrollment; sample size; clinical characteristics of the included patients; study design; the number of excluded patients if specified; statistical analyses utilized; duration of follow-up and number of subjects lost to follow-up; outcome of interest; prognostic variables assessed in each study and quality of the regression model [20,21,22]. When data were available tests required for completion of the tables were performed. To summarize the results regarding secondary prophylaxis, the software Comprehensive Meta-Analysis Version 2.2.048 was used.

("leishmaniasis, visceral"[MeSH Terms] OR ("leishmaniasis"[All Fields] AND "visceral"[All Fields]) OR "VL "[All Fields] OR ("leishmaniasis"[All Fields] AND "visceral"[All Fields]) OR "leishmaniasis, visceral"[All Fields]) AND ("hiv infections"[MeSH Terms] OR ("hiv"[All Fields] AND "infections"[All Fields]) OR "hiv infections"[All Fields]) AND ("recurrence"[MeSH Terms] OR "recurrence"[All Fields] OR "relapse"[All Fields] OR follow-up studies OR prognosis* OR Recurrences OR Relapse OR Relapses OR Recrudescence OR Recrudescence OR Prospective Studies OR monitor*)

Figure 1. Terms used in Medline/PubMed search.
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Results

Our selection process is illustrated in Figure 2. Of 178 articles, 136 were excluded because they did not meet the inclusion criteria following reading of titles and/or abstracts. Twenty more articles were excluded after reading the entire article: six analyzed less than ten patients [23,24,25,26,27,28,29], one was a review [30], and thirteen did not evaluate the risk on relapse of different predictors [3,28,31,32,33,34,35,36,37,38,39,40,41]. Four studies [42,43,44,45] were excluded because they included cases published elsewhere [10,46,47]. Thus, 18 studies (Table S1) satisfied the specified inclusion and exclusion criteria and constituted the basis of this investigation.

Studies and Patients

Table S1 summarizes the characteristics of the 1017 patients encompassed by the 18 included studies. The year of study publication ranged from 1989 to 2008. The design of 8 of the studies examined was prospective. Fourteen studies were reported in Spain, two in Italy, and one in Ethiopia and one in France. Eight studies had an enrollment period exclusively after 1996, when HAART became available. Twelve papers stated the proportions of patients receiving HAART involving two nucleoside reverse transcriptase inhibitors and one or two protease inhibitors or non-nucleosides reverse transcriptase inhibitors at VL diagnosis or at relapse or both.

A large proportion of the patients in these studies (87.7%) were male and most were young adults; the median or mean ages reported varied from 27 to 37 years (Table S2). In the 14 studies in which patients' presumed transmission route was known, 72.3% (420/581) of the infections were likely due to intravenous drug use. The median CD4+ T lymphocyte count ranged from 11 to 82 cells/mL. Most patients had an AIDS-defining condition [48] at the time of VL diagnosis (332/572, 58% of patients).

In the majority of the studies, the diagnosis of VL was established by direct demonstration of amastigotes (by cytological study of Wright stains) or by the observation of promastigote growth in samples cultured in specific media. In one study [49],

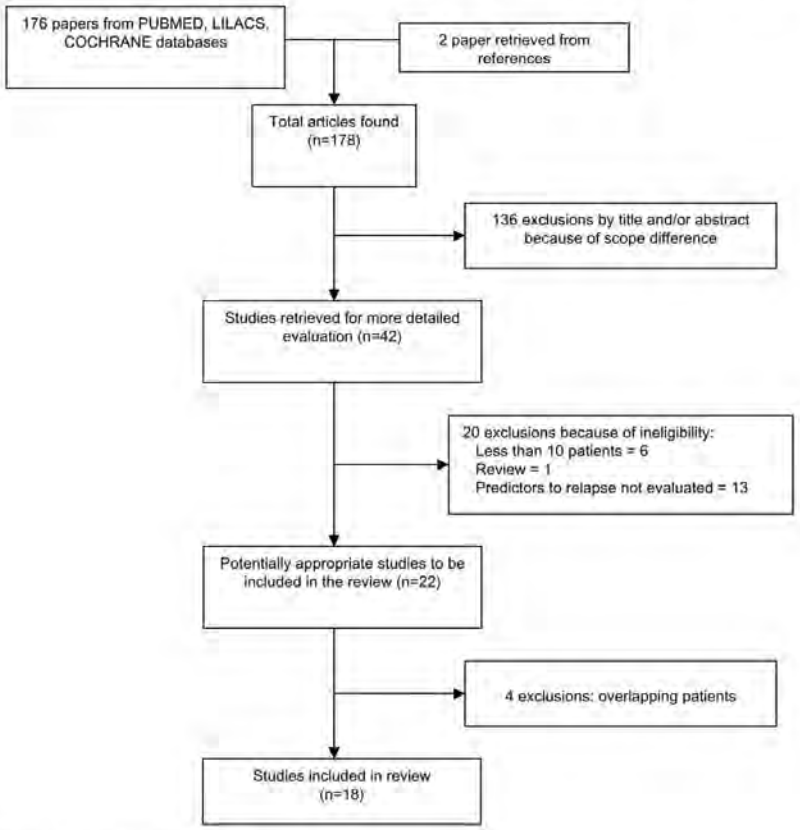

Figure 2. Study selection process.
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the VL diagnosis was supported either by positive results from *Leishmania*-specific PCR (polymerase chain reaction) of peripheral blood or bone marrow samples. Three studies [47,50,51] also included patients diagnosed by serologic tests (direct agglutination, indirect immunofluorescence or rK-39 dipsticks).

The drug used in the treatment of the primary episode of VL was reported for 89% of the treated patients. Of this total, 73.4% of cases (733 patients) were treated with pentavalent antimonial drugs, 12.4% with amphotericin B deoxycholate (124 patients), and 2.1% (21 patients) received amphotericin in lipid formulations. A minority of patients (1.2%) received pentamidine isethionate and three papers included patients treated with miltefosine [47] or unconventional regimens such as a combination of allopurinol with an azole compound [50,52]. A test of cure (staining with Giemsa stain and parasite culture or PCR) at the end of treatment was carried out in 8 of 18 studies. In most of these studies, this control was performed for patients whose clinical response was uncertain. Secondary prophylaxis for leishmaniasis was reported in eleven studies.

Three studies explored the impact of mono or dual antiretroviral therapy at VL diagnosis [47] or during the follow-up [50,53] on relapse. Only one [47] of these studies demonstrated a reduction in relapse rate compared with patients who did not undergo retroviral therapy. Similarly, only one [49] of four studies [10,49,51,54] that followed patients on HAART at VL diagnosis reported a reduction in relapse rate. HAART use on follow-up has also been studied in relation to risk of relapse and none of the five [9,51,52,54,55] studies showed reduction on VL relapse rate.

Two studies [52,54] that evaluated VL prophylaxis without specifying the drug used noted a significant reduction in relapse. In a report of ten cases, Bossolasco et al. [55] showed that the relapse rate in patients groups with and without prophylaxis were 60% and 100%, respectively, but this difference did not reach statistical significance. Three studies evaluated specific prophylactic regimens (antimony compounds [46,50] and liposomal amphotericin [50]) and demonstrated reduction on VL relapse. Although the confidence intervals did not reach statistical significance, another author [56] concluded that lipid-complexed amphotericin prophylaxis also reduced the relapse rate. Finally, Laguna et al. [57] showed a trend towards ($p = 0,08$) a reduction in VL relapse rate following treatment with pentamidine prophylaxis. A meta-analysis of results from all studies evaluating the impact of secondary prophylaxis is shown in Figure 3. This analysis could consistently demonstrate that secondary prophylaxis reduces VL relapse rate.

CD4+ lymphocyte count at VL diagnosis and follow-up has been studied in relation to risk of relapse. Nine articles [10,11,12,46,50,51,52,55,58] compared CD4+ lymphocyte cell counts at VL diagnosis between relapsing and non-relapsing patients as a continuous variable. Neither of these studies showed significant differences between these two groups. On the other hand, two studies [47,49] that compared relapse rate between patients with CD4+ count at VL diagnosis as a dichotomic variable (above and below than 100 cell/mL) noted that the arms with higher CD4+ counts had lower relapse rate. Similarly, an increase in CD4+ lymphocyte count at follow-up was protective against VL relapse in 5 of 7 studies [10,11,49,55,58]. In another study [12], univariate analyses of CD4+ counts at follow-up revealed a trend towards a reduction in relapse ($p = 0.09$).

Other variables explored in relation to relapse are shown in Table S3. Factors such as age, route of HIV transmission, history of intravenous drug use, HIV viral load at VL diagnosis, various clinical findings, specific anti-*Leishmania* treatments given, time from VL diagnosis to the introduction of protease inhibitor therapy, HAART compliance, the presence of an AIDS-defining disease before VL diagnosis and the presence of serum anti-*Leishmania* antibodies were not substantially different between relapsing and non-relapsing patients. Tuberculosis co-infection [47], hepatitis C virus co-infection [49] and an incomplete course of VL treatment [52] were evaluated in multivariate analysis and showed a statistically significant association of these conditions with the occurrence of relapse. Previous VL episodes were identified as risk factors for relapse in 3 studies, two of which were multivariate analyses.

Prognostic Variables and Statistical Analysis

The statistical quality and the presentation of methods and results in many studies were poor. In nine studies, the Kaplan-Meier method was used in a univariate survival analysis to

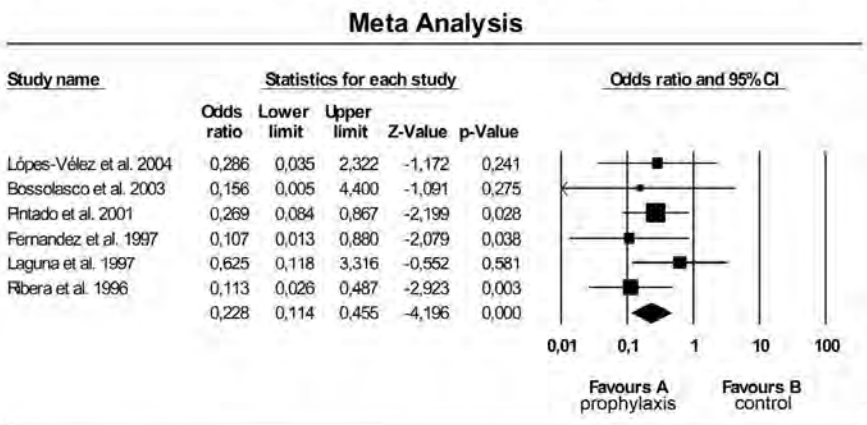

Figure 3. Meta-analysis of secondary prophylaxis results. Footnote: $I^2 = 0\%$ Egger test for publication bias was negative, $p = 0.76$.
doi:10.1371/journal.pntd.0001153.g003

analyzed VL relapse. Three prospective studies and two retrospective cohort studies employed Cox regressions for multivariate analysis of independent predictors. One study randomized patients to compare prophylaxis (liposomal amphotericin versus no treatment) and performed multivariate analysis to compare relapse rates by logistic regression, including some predictors as covariates. None of these six studies mentioned collinearity assessment (i.e., a high degree of correlation between 2 predictive variables) or developed a risk score for relapse based on their multivariable results. Also, none of the multivariate analyses reported a goodness-of-fit test of their models. Other studies analyzed isolated relapse predictors by univariate association tests in series of prospective or retrospective cases or in intervention studies.

Discussion

The present study is the first systematic review of predictors of VL relapse in HIV-infected patients. Our main conclusions are that VL relapse in HIV-infected patients receiving HAART is high and that secondary prophylaxis provides some protective effect but does not completely prevent the occurrence of relapse. We found that patients who did not relapse showed significantly higher CD4+ count at follow-up than patients with relapsing course. Our analysis also suggests that CD4+ count greater than 100 cell/mL at VL diagnosis reduces the odds of relapse. Unlike other opportunistic infections there are some reports of VL relapse in patients with a CD4+ count greater than 200 cell/mL in Ethiopia, and rarely in Europe [9]. This evidence shows that factors other than a CD4+ cell increase are involved in VL control. A threshold for safely discontinuing of secondary prophylaxis has not been established because of these uncertainties.

Most cases reported showed severe reductions in T cells. It could indicate that VL affects HIV-1 patients who exhibit a significant disturbance of cellular immunity; however, VL by itself may reduce CD4+ lymphocyte counts [59]. On the other hand, a CD4+ count greater than 100 cell/mL at VL diagnosis is a potential protective factor against relapse, although the analysis of this beneficial effect may be complicated by the immunosuppression of many the patients included in the studies. When analyzing the CD4+ count range and number of patients with CD4+ counts of greater than 100 cell/mL in the two studies [47,49] demonstrating an association between higher baseline CD4+ counts and reduced VL relapse, it is possible to speculate that studies that did not demonstrate an influence of CD4+ cells had few patients with CD4+ counts of greater than 100 cell/mL. Studies using animal models reported that CD4+ cells are responsible for the initial control of parasite proliferation and dissemination [60]. Thus, a low initial CD4+ count might allow a wide dissemination of the parasite throughout the phagocytic mononuclear system at the beginning of infection, increasing the number of sites that could harbor quiescent parasites (so-called "sanctuaries") [61].

Relapses of VL are suggested to occur mainly in individuals with poor responses to antiretroviral treatment who have no improvement in CD4+ counts [11,12,58,62], with a few exceptions [9,47]. The evolution of patients who acquire VL and thereafter show a significant increase in CD4+ counts while on HAART is currently receiving attention [47 50 51 52]. It has already been established that the outcome of VL is not influenced by humoral immunity but appears to be regulated by CD4+ T helper cells with different patterns of cytokine activity [63]. Protective immunity can be attributed to T helper (Th)-1 cells, whereas Th-2 cell responses produce IL-4 and IL-10 and facilitate the intracellular survival of the parasite [64]. It might be expected that highly active antiretroviral drug combinations would favor an immunological shift from type 2 to type 1 cytokines in HIV-infected individuals. However, increased CD4+ values in peripheral blood and lymphoid tissues as a result of antiretroviral therapy may have negligible effects on cytokine production during the first 24 weeks [65]. In addition, patients on HAART show an initial increase in the CD4+ memory subset, whereas naive CD4+ cells consistently increase only after 1 year [66].

It is known that HIV patients who are receiving HAART have fewer opportunistic infections and recent data shows that there has been a decline in the incidence of VL after the introduction of HAART [41,54,67,68,69]. HAART seems to be insufficient to prevent VL relapse. Studies in patients receiving HAART showed a relapse rate similar to other studies performed in the pre-HAART era. Only one [49] observational study noted a reduction in the relapse rate among patients on HAART at VL diagnosis. None of the studies reported a statistically significant difference in VL relapse between patients receiving and not receiving HAART on follow-up. These disappointing results so far disagree with a statistically significant association between improvement of CD4+ count at follow-up and reduction of VL relapse. They may be due to the small sample sizes of the studies performed, poor patient adherence to antiviral therapy or insufficient immune response. One possibility to be explored in the future is the role of cytokines [70] influencing the control of VL independently of the CD4+ lymphocyte. The heterogeneity of zymodemes that exhibit different degrees of virulence or parasite burden could contribute to the differences observed in the host immune response and clinical evolution [9]. HAART increases CD4+ count thus influencing the control of VL, but may not be enough in this complex scenario created by the co-infection HIV and *Leishmania*. Fernandéz-Cotarelo et al. [54] and others [41] have shown a decrease in the number of new episodes of VL in HIV-infected patients receiving HAART but also a tendency toward VL relapse. According to these authors the high rates of relapse could be explained by the increased patient survival that results from effective antiretroviral therapy.

Previous episodes of VL were strongly associated with relapse. Also in agreement with the immune-inflammatory theory, it was hypothesized that the enhancement of the Th-2 response following one early relapse could complicate or prevent the later control of *Leishmania* infection [54].

Secondary prophylaxis seemed to only partially protect against relapse. Some of studies that observed a reduction in VL relapse following the use of secondary prophylaxis had few patients on HAART, which may not reflect the current reality. Data analysis suggests that the small sample sizes and heterogeneity of regimens used make the results less robust. Nevertheless, the evaluation of these studies through meta-analysis indicates a clear benefit of secondary prophylaxis in reducing VL relapse. Based on six studies whose data were available, the average relapse rate in patients not receiving secondary prophylaxis was 67%, while in the secondary prophylaxis arm, the relapse rate was 31%. Given this difference, the estimated total sample size needed for a study with 80% power would be 70 patients. Three out of the six studies examining secondary prophylaxis were not able to demonstrate statistical significance, possibly because of small sample sizes. It is important to emphasize that despite the heterogeneity of prophylaxis regimens used, statistical results are positively homogeneous in meta-analysis.

Thresholds for safe discontinuation of secondary prophylaxis for Spanish patients have been suggested to be CD4+ counts of 200 [71] and 350 cells/mL [11]. Differently of the European experience, one Ethiopian study [47] has shown that many patients suffering relapse (11 from 39 cases) had a CD4+ count above 200 cells/mL before relapse. These data may suggest that *L. donovani*, the predominant

causative agent of VL in east Africa and south Asia, is a more virulent and anthroponotic species than *L. infantum*. Another plausible explanation for this difference may be the influence of other variables that can affect the host immune response such as nutritional status and the presence of other infections and co-morbidities.

It has been postulated that the maintenance of an undetectable viral load protects against the development of VL [17] and that a high viral load could predict a weak response to antiparasitic treatment [12] although there are contradictory reports on this point [54,72]. None of the papers reviewed here linked HIV load by PCR at VL diagnosis with relapse. On the other hand HIV load by PCR at follow-up was statistically related to relapse in one [58] of four studies that evaluated this variable in a univariate analysis. These observations support the idea that a sustained immunological response is more important than a virological response to cure VL in HIV-infected patients.

It is important to note that a wide range of therapeutic drugs were utilized for the treatment of VL in the studies we have reviewed. There was no notable difference in the relapse rate with regard to specific VL treatment used (all analyzed in univariate analysis); however only four studies explored this association and most of them included a limited number of patients and only two [11,73] involved randomly assigned patients. Few comparative clinical studies have been conducted of the efficacy of treatment for HIV VL co-infection outside the Mediterranean area. In some instances [74,75], the development of drug resistance could contribute to therapeutic failure and the relapsing course observed in HIV-infected patients. These observations do not allow us to refute the influence of anti-parasite treatment on relapse outcome.

Study Limitations

Although we have made an extensive review, our analysis includes studies with different definitions of cure and different lengths of follow-up. Cure is seldom defined parasitologically in these studies and the difference between treatment failure and relapse is arbitrary in some studies. It is possible that some episodes of relapse in the group of patients in which parasitological cure were not documented by bone marrow examination were treatment failures rather than relapses. Moreover, re-infection was not distinguished from relapse in any paper. There is a high degree of heterogeneity in the evaluated populations as shown by the wide range of reported mortality (6.5% to 83.8%), treatment failure (0 to 47.6%) and relapse rates (20% to 70%). These studies included patients with different degrees of immunosuppression, and different treatment and prophylaxis regimens. Also, there are differences in the study designs, the types of statistical methods used and the prognostic variables included in analysis. These variations may have resulted in patient selection bias or low statistical power, thus hampering a meta-analysis of all studied predictors of relapse. In spite of these limitations, we believe that the meta-analysis results of secondary prophylaxis are consistent, considering the available evidence. In addition, the quality of published reports was heterogeneous and usually poor. Despite these limitations, this review may assist clinicians in making decisions and may also help in the design of future studies.

Conclusion

The results of this systematic review suggest there are identifiable predictive factors of VL relapse, such as previous episodes of VL relapse and lack of recovery of CD4+ lymphocyte numbers after primary visceral leishmaniasis. HAART did not produce the anticipated decrease in the incidence of VL relapses and more data is needed in order to better assess the evolution of VL in the HAART era. In contrast, secondary prophylaxis was shown to be protective against relapse. CD4+ count below 100 cells/mL at the time of VL primary diagnosis is a potential predictor of relapse.

Based on these observations, a high-risk population might be identified and such patients might then be eligible for secondary prophylaxis. Strong surveillance will certainly contribute to improved data quality for decision-makers in this complex scenario. Randomized trials to compare the efficacy of different drugs and their role either in treatment or in prophylaxis are required.

Supporting Information

Table S1 Parasitological control. Identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples dAmB: amphotericin B deoxycholate LAmB: liposomal amphotericin LipAmB: amphotericin B lipid complex. PA: Pentavalent antimonial compounds **SD**: standard deviation **IRQ**: interquartile range **x̃**: median **μ**: mean.
(DOC)

Table S2 VL: Visceral leishmaniasis **Parasitological confirmation**: identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples #**Serology confirmation**: *Leishmania* direct agglutination positive §**Biologic confirmation**: identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples or Leishmania-specific PCR on peripheral blood/bone marrow **dAmB**: amphotericin B deoxycholate **LAmB**: liposomal amphotericin B **LipAmB**: amphotericin B lipid complex **PA**: Pentavalent antimonial compounds **Hemo**: transfusion route **IDU**: intravenous drug user **HETERO**: heterosexual contacts **HOMO**: men who have sex with men **sexual**: heterosexual or homosexual contacts **SD**: standard deviation **IRQ**: interquartile range ‡ if the information was available x̃: median μ: mean.
(DOC)

Table S3 Yes: positive association **No**: negative association **VL**: Visceral leishmaniasis **HAART**: highly active antiretroviral therapy **HVC**: hepatitis C virus * multivariate analysis.
(DOC)

Author Contributions

Conceived and designed the experiments: GFC MRdS AR. Performed the experiments: GFC MRdS. Analyzed the data: GFC MRdS. Wrote the paper: GFC MRdS. Paper review: AR.

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Table 1. Visceral leishmaniasis relapse in HIV-1 infected patients: characteristic of studies and outcomes

Reference	*Period of enrollment and Country*	*Sample size*	*Study design*	*Cure assessment*	*Median or μ mean length of follow-up (range or SD) month*	*Patients lost to follow-up (%)*	*Treatment failure %*	*Mortality rate (%)*	*Secondary prophylaxis (number of patients: prophylactic regimen)*	*Relapse rate(%)*	*Statistical analysis*
Ter Horst, 2008	2003 to 2006, Ethiopia	161 without antiretroviral therapy	Retrospective cohort single center	Clinical improvement and parasitological control (some patients)	3,1 (0-36.5)	120 (74.7)	Not informed	11/161 (6.8)	All patients: no prophylaxis	Not informed	Multivariate
		195 with antiretroviral therapy			7,1 (0.5-33.5)	37 (19.1)		28/195 (14.4)		43/195 (22)	
Bourgeois, 2008	1995 to 2004, France	27	Prospective observational two centers	Not informed	51 (5-108)	2 (7.4)	Not informed	7/27 (26)	All patients: dAmB 0.6 to 0.7 mg/kg or LAmB 3-4mg/kg twice monthly or monthly	16/27 (59.3)	Multivariate
Molina, 2007	2001 to 2005, Spain	15	Prospective non-controlled single center	Clinical improvement and parasitological control (some patients)	14 (5-44)	2/15 (13.3)	0	1/15 (6.6)	All patients: LAmB 5mg/kg every 3 weeks (replaced by miltefosina after 2003 in three patients)	3/15 (20)	Univariate
Pasquau, 2005	1988 to 2001, Spain	155	Retrospective observational multicenter	Clinical improvement	8.4 (IQR 1,8-19.4)	Not informed	20%	32/155 (21)	34 patients: no prophylaxis	37/96 (38.5)	Multivariate
									30 patients: PA; 2 patients: dAmB; 20 patients: pentamidine; 4 patients: LAmB; 6patients: others agentes		
Mira, 2004	1989 to 2002, Spain	21 without secondary prophylaxis	Retrospective cohort two centre	Not informed	25 (2−61)	4 (12.9)	Not informed	10/31 (32.2)	21 patients: no prophylaxis	8/21 (38)	Univariate
		10 with secondary prophylaxis			30 (4−53)				10 patients: secondary prophylaxis with drug not informed	1/10 (10)	
López-Vélez, 2004	1997 to 1999, Spain	17	Prospective comparative multicenter randomized for prophylaxis use comparison	Clinical improvement and parasitological control	12	Not informed	0	Not informed	9 patients: no prophylaxis	4/8 (50)	Multivariate
									8 patients: LipAmB 3mg/kg/d every 3 weeks	7/9 (88.8)	
Fernandéz-Cotarelo, 2003	1994 to 2000, Spain	34	Retrospective observational single center	Not informed	Not informed	Not informed	Not informed	Not informed	Number of patients under secondary prophylaxis not informed.	13/34 (38.2)	Univariate
Bossolasco, 2003	Not informed, Italy	10	Prospective observational two centers	Clinical improvement	8,9 (1.5-60)	1/10 (10)	Not informed	0	4 patients: no prophylaxis	4/4 (100)	Univariate
									5 patients: prophylaxis with LAmB 3mg/kg once a month	3/5 (60)	
Casado, 2001	1996 to 1997, Spain	10	Prospective observational single center	Not informed	31	Not informed	Not informed	Not informed	All patients: : no prophylaxis	7/10 (70)	Univariate
Pizzuto, 2001	1997 to 1999, Italy	10	Prospective observational multicenter	Not informed	22 (7.5-27.5)	Not informed	Not informed	3/10 (30)	All patients: : no prophylaxis	6/10 (60)	Univariate
Pintado, 2001	1974 to 1997, Spain	80	Retrospective observational single center	Clinical improvement and parasitological control (some patients)	μ 13.8 (3–44)	16/80 (20)	23.3	43/80 (53.7)	33 patients: no prophylaxis	22/33 (66.7)	Multivariate
									9 patients: LAmB; 7 patients: PA; 3 patients: allopurinol, 1 patient: pentamidine	7/20 (35)	
Berenguer, 2000	1998 to 2000, Spain	15	Prospective observational single center	Not informed	15.8 (1-22)	0	Not informed	Not informed	All patients: : no prophylaxis	3/15 (20)	Univariate
Villanueva, 2000	1996 to 1999, Spain	32	Prospective observational single center	Clinical improvement and parasitological control	μ 14,7 (3-34)	10/32 (31)	6.3	3/32 (9.4)	All patients: no prophylaxis	5/20 (25)	Univariate
Laguna, 1999	1994 to 1996, Spain	89	Prospective, multicenter, randomized for treatment comparison	Clinical improvement and parasitological control	10.8	5/89 (5.6)	11.7(PA) 6.7 (dAmB	10/89 (11.2)	All patients: no prophylaxis	19/48 (39.6)	Univariate
Laguna, 1997	1986 to 1994, Spain	43	Retrospective single center	Clinical improvement and parasitological control	μ 9.9 ± 3.3	4/43 (9.3)	29.4	14/43 (32.6)	10 patients: no prophylaxis	5/10 (50)	Univariate
					μ 10.4 ± 3.3				13 patients: pentamidine 4mg/kg once month	5/13 (38)	
Fernandez, 1997	1992 to January,1996 Spain	31	Retrospective single center	Clinical improvement	μ 10.9 ± 5	Not informed	47.6 (PA) 3.3 (dAmB)	26/31 (83.8)	6 patients: no prophylaxis	4/6 (66.7)	Univariate
									17 patients: prophylaxis with drug not informed	3/17 (17.4)	
Ribera, 1996	1988 to 1995, Spain	46	Retrospective single center	Clinical improvement and parasitological control (some patients)	Not informed	Not informed	Not informed	3/46 (6.5)	20 patients: no prophylaxis	13/20 (65)	Multivariate
									9 patients: Allopurinol 900mg/day	5/9 (6.6)	
									17 patients: PA 850mg once a month	3/17 (17.6)	
Montalban, 1989	1983 to 1988, Spain	16	Retrospective multicenter	Not informed	12 (0.5-60)	Not informed	12.5	6/16 (37.5)	All patients: no prophylaxis	5/14 (35.7)	Univariate

Parasitological control: identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples dAmB: amphotericin B deoxycholate LAmB: liposomal amphotericin LipAmB: amphotericin B lipid complex
PA: Pentavalent antimonial compounds **SD**: standard deviation **IRQ**: interquartile range : median **μ**: mean

Table 2. Visceral leishmaniasis relapse in HIV-1 infected patients: characteristic of population

Reference	Median or μmean age at primary VL diagnosis (range or SD) years	Gender male (%)	Diagnosis of AIDS prior VL (%)	Patients with each risk factor for HIV-1 transmission (%)	CD4+ baseline cell count median or μmean (range or SD cells/mL)	Patients with CD4+ < 200 cells/mL at primary VL diagnosis (%)	Visceral leishmaniasis diagnosis	Treatments of visceral leishmaniasis used (number of patients♯)	Patients under HAART at primary VL diagnosis (%)	Patients under HAART at VL relapse (%)
Ter Horst, 2008	Without antiretroviral therapy: 31.3(20-50)	158 /161 (98.2)	Not informed	Not informed	Not informed	71/ 161 (44,1)	Clinical signs and serology # or parasitological confirmation	PA 20mg/kg 30 days (323) or miltefosina 100mg/day 28 days	Not informed	Not informed
	With antiretroviral therapy: 33,5(18-60)	176/195 (90.5)				166/195 (85,6)				
Bourgeois, 2008	35 (30-51)	22/27 (81.5)	12/27 (44)	IDU: 12 (44.4) Homo: 6 (22.3) Hetero: 8 (29.6) Hemo: 1 (3.7)	51 (4 to 322)	26/27 (96.3)	Clinical signs and biological§ confirmation	dAmB 0.8-1g cumulative amount or Ant (26) or PA 20mg/kg 30 days (1)	12/27 (44)	Not informed
Molina, 2007	36 (26-53)	14/15 (93.3)	7/12 (58.3)	IDU: 13 (86,7) Hetero 2 (13,3)	82 (4-210)	12/12 (100)	Parasitological confirmation	LAmB 4mg/kg for 5 consecutive days and once per week thereafter for 5 more weeks (17)	5/12 (42)	8/9 (88.8)
Pasquau, 2005	32 (IRQ 30–37)	129/155 (83)	94/155 (61)	IDU: 116 (75) Homo: 8 (5) Hetero: 20 (13) Unknown: 11 (7)	56 (20-120)	Not informed	Clinical signs and parasitological confirmation	PA 20mg/kg/d at least 21 days (139), dAmB - cumulative dose t least 1.5g (7), lipid formulations of amphotericin - cumulative dose at least 1.5g (5), pentamidine 4mg/kg/d 2 weeks (1) or fluconazole + allopurinol (2)	24/155 (15)	11/37 (30)
Mira, 2004	μ 33 (24-57)	19/21 (90)	8/10 (80)	Not informed	Not informed	Not informed	Clinical signs and parasitological confirmation	PA 20mg/kg/day 28 days (13) or pentamidine 4mg/kg/day 28 days (2) or dAmB – 0.7mg/kg/d 28 days (5) or LAmB 2.5-4 mg/kg/d 10 days (9) or LipAmB 5mg/kg/day 14 days (5)	0	19/21 (90.5)
	μ 34 (26-37)	10/10 (100)	16/21 (76)						0	10/10 (100)
López-Vélez, 2004	μ 37+ 5	7/8 (87.5)	7/8 (87.5)	Not informed	Not informed	Not informed	Clinical signs and parasitological confirmation	Not informed	Not informed	8/9 (88.9)
	μ 5+ 6	9/9 (100)	4/9 (44.4)							8/8 (100)
Fernandéz-Cotarelo, 2003	μ 34.6 (range: 27–60)	31/34 (91.2)	Not informed	IDU: 27 (77) Homo: 4 (11,2) Hetero: 2 (5.9) Unknown: 1 (2.9)	Not informed	Not informed	Clinical signs and parasitological confirmation	Not informed	7/34 (20.6)	10/13 (76.9)
Bossolasco, 2003	37 (30-42)	8/10 (80)	Not informed	IDU: 7 (70). Hetero: 2 (20) Homo: 1 (10)	42 (5-246)	9/10 (90)	Clinical signs and parasitological confirmation	LAmB 3mg/kg/day on days 1-5 and once weekly thereafter between 17-66 days (10)	4/10 (40)	5/7 (71.4)
Casado, 2001	μ 34 (31-38)	6/10 (60)	Not informed	IDU: 6 (60) Sexual: 4 (40)	70 (3–156)	10/10 (100)	Clinical signs and parasitological confirmation	PA 20mg/kg 28 days or dAmB 0.7 mg/kg/day 28 days	0	10 (100)
Pizzuto, 2001	32 (27-45)	8/10 (80)	8/10 (80)	IDU: 8 (80) Hetero: 2 (20)	70 (4-190)	10/10 (100)	Clinical signs and serology # or parasitological confirmation	PA (4) or LAmB (3) or dAmB (3) ("at standard doses")	5/10 (50)	7/10 (70)
Pintado, 2001	μ 33.2 + 8.2	64/80 (80)	43/80 (53.7)	IDU: 63 (78.7) Homo: 6 (7.5) Hetero: 6 (7.5) Perinatal: 1 (1.3) Unknown: 2 (2.5)	μ 90 (3–470)	61/70 (87.1)	Clinical signs and serology # or parasitological confirmation	PA 20 mg/kg/day - with a maximum daily dose of 850 mg) for 3–4 weeks (51) or dAmB 0.5–1 mg/day for 3–4 weeks (17) or Allopurinol + azoles compounds (4)	2/73 (2.7)	Not informed
Berenguer, 2000	37 (24-47)	9/15 (60)	7/15 (46.7)	IDU: 9 (60) Homo: 2 (13.3) Hetero: 3 (20) Unknown: 1 (6.7)	77 (3-215)	13/14 (92.3)	Clinical signs and parasitological confirmation	PA 20 mg/kg per day for 28 days or LAmB total of 10 doses of 4 mg/kg per day given on days 1 to 5, 10, 17, 24, 31 and 38.	Not informed	15/15 (100)
Villanueva, 2000	μ 32.6 + 5,4	20/32 (62.5)	Not informed	Not informed	50 (1-200) with HAART 69 (27-166) without HAART	Not informed	Clinical signs and parasitological confirmation	PA 20mg/kg/d 28 days (23) or LipAmB 3mg/kg/d 5-10 days (3) or LAmB 4mg/kg twice weekly 6 weeks (1)	Not informed	5/5 (100)
Laguna, 1999	32 (19–64)	76/89 (85)	56/89 (63)	IDU: 61 (69) Sexual: 16 (18) Others :12 (13)	20 (0-231)	Not informed	Clinical signs and parasitological confirmation	PA 20 mg/kg/day (44) or dAmB - 0.7 mg/kg per day (45), both for 28 days	0	0
Laguna, 1997	Not informed	42/43 (98)	29/43 (67)	IDU: 35 (81) Sexual: 7 (16%) Others :1 (2)	μ 10 ±3.3	Not informed	Clinical signs and parasitological confirmation	PA low dose:< 20 mg/kg/day 21 days (17) or PA high dose: ≥20 mg at least 28 days (29) or dAmB (1) or LAmB (4)	0	0
Fernandez, 1997	μ 34,3+ 5,1	30/31 (96.7)	18/31 (58.1)	IDU :21 (67.7) Homo: 6 (19.4) Hetero: 4 (12.9)	μ 36,9 ± 27,9	31/31 (100)	Clinical signs and parasitological confirmation	PA 20 mg/kg/d - maximum 850mg/d 21 days (21) or dAmB 1-1.5 g cumulative amount - 0.5 mg/kg per day (20)	0	0
Ribera, 1996	No prophylaxis 27 (20-64)	Not informed	9/20 (45)	IDU: 9 (45)	35 (2-125)	Not informed	Clinical signs and parasitological confirmation	PA 850mg/day 21 days or dAmB (52)	0	0
	Allopurinol prophylaxis 28 (22-29)		6/9 (67)	IDU: 5 (55)	11 (2-42)					
	PA prophylaxis 29 (21-36)		3/17 (18)	IDU: 13 (76)	34 (8-268)					
Montalban, 1989	Not informed	14/16 (87.5)	5/16 (31)	IDU: 15 (94) Homo:1 (6)	Not informed	Not informed	Clinical signs and parasitological confirmation	PA (16)	0	0

VL: Visceral leishmaniasis **Parasitological confirmation:** identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples **#Serology confirmation**: *Leishmania* direct agglutination positive **§Biologic confirmation**: identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples or Leishmania-specific PCR on peripheral blood/bone marrow **dAmB**: amphotericin B deoxycholate **LAmB**: liposomal amphotericin B **LipAmB**: amphotericin B lipid complex **PA**: Pentavalent antimonial compounds **Hemo**: transfusion route**IDU**: intravenous drug user **HETERO:** heterosexual contacts **HOMO:** men who have sex with men **sexual**: heterosexual or homosexual contacts **SD:** standard deviation **IRQ:** interquartile range ♯if the information was available : median μ: mean

Table 3. Visceral leishmaniasis relapse in HIV-1 infected patients: variables investigated

VARIABLES	*Ter Horst, 2008*	*Bourgeois, 2008*	*Molina, 2007*	*Pasquau, 2005*	*Mira, 2004*	*López-Vélez, 2004*	*Fernandéz-Cotarelo, 2003*	*Bossolasco, 2003*	*Casado, 2001*	*Pizzuto, 2001*	*Pintado, 2001*	*Berenguer, 2000*	*Villanueva, 2000*	*Laguna, 1999*	*Laguna, 1997*	*Fernandez, 1997*	*Ribera, 1996*	*Montalban, 1989*
Tuberculosis co-infection	Yes *																	
VL previous	Yes *											No	Yes				Yes *	
Age		No		No				No		No		No						
Female gender		No		Yes *	No			No		No		No						
Intravenous drug use		No	No	No	No			No		No		No						No
Homosexual contact		No										No						
AIDS previous to VL		No	No	No	No					No	No *	No					No *	No
PCR HIV load at baseline		No	No		No			No	No	No			No					
PCR HIV load at follow-up					Yes			No				No	No					
Antiretroviral therapy use at VL diagnosis	Yes *																	
Antiretroviral therapy use at follow-up											No *				No			
HAART use at VL diagnosis		Yes	No				No			No								
HAART use at follow-up				No			No	No		No			No					
HAART compliance					No													
CD4+ count at VL diagnosis			No	No	No			No	No	No	No *	No					No *	
CD4+ count < 100 cell/mL at VL diagnosis	Yes *	Yes *											No					
CD4+ count < 200 cell/mL at VL diagnosis			No															
CD4+ count at follow-up		Yes	Yes		Yes			Yes	No			Yes	No					
HCV co-infection		Yes *																
Sexual transmission			No															
Completed therapy for VL				Yes *														
Specific anti-*Leishmania* treatment given												No	No	No				
Secondary prophylaxis use (non specificated regimen)				Yes *				No								Yes		
Antimonial secondary prophylaxis											Yes *						Yes	
Liposomal amphotericin secondary prophylaxis											Yes *							
Pentamidine secondary prophylaxis															No			
Amphotericin complex lipid secondary prophylaxis						No *												
Clinical findings (hepatomegaly, splenomegaly, anemia, leucopenia, thrombocytopenia)				No														
Time from de VL diagnosis to the introduction of protease inhibitors therapy									No									
Anti-*Leishmania* antibody positive								No		No	No *						No	

Yes: positive association **No:** negative association **VL:** Visceral leishmaniasis **HAART**: highly active antiretroviral therapy **HVC:** hepatitis C virus ***** multivariate analysis

ARTIGO 2

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The Diagnostic Accuracy of Serologic and Molecular Methods for Detecting Visceral Leishmaniasis in HIV Infected Patients: Meta-Analysis

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PLoS NEGLECTED TROPICAL DISEASES

The Diagnostic Accuracy of Serologic and Molecular Methods for Detecting Visceral Leishmaniasis in HIV Infected Patients: Meta-Analysis

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Abstract

Background: Human visceral leishmaniasis (VL), a potentially fatal disease, has emerged as an important opportunistic condition in HIV infected patients. In immunocompromised patients, serological investigation is considered not an accurate diagnostic method for VL diagnosis and molecular techniques seem especially promising.

Objective: This work is a comprehensive systematic review and meta-analysis to evaluate the accuracy of serologic and molecular tests for VL diagnosis specifically in HIV-infected patients.

Methods: Two independent reviewers searched PubMed and LILACS databases. The quality of studies was assessed by QUADAS score. Sensitivity and specificity were pooled separately and compared with overall accuracy measures: diagnostic odds ratio (DOR) and symmetric summary receiver operating characteristic (sROC).

Results: Thirty three studies recruiting 1,489 patients were included. The following tests were evaluated: Immunofluorescence Antibody Test (IFAT), Enzyme linked immunosorbent assay (ELISA), immunoblotting (Blot), direct agglutination test (DAT) and polimerase chain reaction (PCR) in whole blood and bone marrow. Most studies were carried out in Europe. Serological tests varied widely in performance, but with overall limited sensitivity. IFAT had poor sensitivity ranging from 11% to 82%. DOR (95% confidence interval) was higher for DAT 36.01 (9.95–130.29) and Blot 27.51 (9.27–81.66) than for IFAT 7.43 (3.08–1791) and ELISA 3.06 (0.71–13.10). PCR in whole blood had the highest DOR: 400.35 (58.47–2741.42). The accuracy of PCR based on Q-point was 0.95; 95%CI 0.92–0.97, which means good overall performance.

Conclusion: Based mainly on evidence gained by infection with *Leishmania infantum chagasi*, serological tests should not be used to rule out a diagnosis of VL among the HIV-infected, but a positive test at even low titers has diagnostic value when combined with the clinical case definition. Considering the available evidence, tests based on DNA detection are highly sensitive and may contribute to a diagnostic workup.

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Introduction

Leishmaniasis gained higher clinical importance in individuals infected with HIV-1 (human immunodeficiency virus type-1) as an opportunistic infection in areas where both infections are endemic. In immunocompromised patients, the clinical course of the disease is even less specific and can be masked by other associated opportunistic infection [1]. Co-infected patients classically present a chronic clinical course and high rate of treatment failure [2]. There is no doubt that the actual number of documented cases of co-infection is underestimated due to the various problems in recognition, diagnosis and reporting of either HIV-1 infection, or leishmaniasis or both, in the setting of developing countries [3].

Parasitological diagnosis remains the gold standard in the diagnosis of leishmaniasis mainly because of its high specificity [4]. Demonstration of *Leishmania* parasites in bone marrow aspirate or in other biologic specimens, either by visualization or culture, is also the most reliable diagnostic technique in the setting of HIV co-infection. However, microscopic examination requires invasive procedures and *in vitro* parasite isolation is difficult and time-consuming.

Antileishmanial antibodies have high diagnostic value in immunocompetent patients [5,6] and a wide range of serological methods varying in sensitivity and specificity are available for the VL diagnosis. For immunosupressed individuals, serological investigation is considered not an accurate diagnostic method since a large number of these patients do not harbor antibodies detectable

Author Summary

Human visceral leishmaniasis (VL), a potentially fatal disease, has emerged as an important opportunistic condition in HIV infected patients. In immunocompromised patients, serological investigation is considered not an accurate diagnostic method for VL diagnosis and molecular techniques seem especially promising. Demonstration of Leishmania parasites in bone marrow aspirate or in other biologic specimen, either by visualization or culture, remains the most reliable diagnostic technique in the setting of HIV co-infection. However, these tests are difficult to perform in rural areas and some of them are invasive and carry a risk of complication. This work is a systematic review to evaluate the accuracy of serologic and molecular tests for VL diagnosis in HIV-infected patients. Two reviewers searched the literature, evaluating quality of studies and comparing performance of diagnostic tests. Thirty three studies were included. Most studies were carried out in Europe. Serological tests varied in performance, but with overall limited sensitivity. Based on the evidence, serological tests should not be used to rule out a diagnosis of VL among HIV-patients, but a positive test at even low titers has diagnostic value when combined with the clinical case definition. Tests based on DNA detection are highly sensitive and may contribute to a diagnostic workup.

by standard techniques based on studies done in Europe [7–9] and in Africa (6). Moreover, there is some doubt whether one serological technique would be superior to the other for the VL diagnosis among HIV-infected patients [8,10–12] and if there is difference in tests performance among global regions.

Over the past 10 years, several molecular techniques targeting various parasite genes have been developed for VL diagnosis. The polymerase chain reaction (PCR) based method is the most common molecular test successfully used and its use looks specially promising in immunosupressed patients [13–16]. This technique has emerged as a more rapid, sensitive, and specific than the traditional diagnostic methods for VL diagnosis [15,17,18].

To our knowledge, antibody detection and molecular tests for the VL diagnosis among HIV-infected patients has not been systematically reviewed and synthesized. We therefore conducted a systematic review to summarize the evidence on diagnostic accuracy (sensitivity and specificity, likelihood ratio, diagnostic odds ratio and Q point from summary ROC curve) of available serological and PCR-based tests, according to the guidelines and methods proposed for diagnostic systematic reviews and meta-analysis [19,20]. The aim of this study is to appraise the diagnostic accuracy of serologic and molecular tests for detecting symptomatic visceral leishmaniasis in patients infected by HIV.

Materials and Methods

Literature Review

Selection was made independently by two reviewers (GFC and MRS) and discrepancies were solved by consensus after discussion. PubMed database search was performed using terms shown in Figure 1. A similar search by using Boolean operators in LILACS database was done.

The selected articles were read in full to confirm eligibility and doubts or disagreements were solved by discussion with a third author (AR). We searched both databases for articles published until 27, July 2011 that reported any available serologic or molecular tests for visceral leishmaniasis diagnosis in HIV-infected individuals over 14 years with symptomatic VL and diagnostic confirmation by examination by parasitological, serologic or molecular tests. No restrictions were made with respect to study design (cross sectional or case control) or data collection (prospective or retrospective). We obtained additional articles by citation tracking of review articles and original articles.

We excluded studies reporting other immune-depressing conditions when co-infected patients with HIV were not identified, series presenting 10 or less patients tested by the index test, review of series of cases and studies where separated results for each serologic test were not presented.

Data Extraction

Data were extracted by one reviewer directly from the full length articles to structured tables containing all the descriptive

("leishmaniasis, visceral"[MeSH Terms] OR ("leishmaniasis"[All Fields] AND "visceral"[All Fields]) OR "visceral leishmaniasis"[All Fields] OR ("visceral"[All Fields] AND "leishmaniasis"[All Fields])) AND ("hiv infections"[MeSH Terms] OR ("hiv"[All Fields] AND "infections"[All Fields]) OR "hiv infections"[All Fields]) AND (agglutination tests OR reagent kits, diagnostic OR antigens, protozoan OR latex fixation tests OR immunology OR diagnosis OR parasitology/methods OR reproducibility of results OR sensitivity and specificity OR genetics OR antibodies, protozoan OR immunoblotting OR enzyme-linked immunosorbent assay OR fluorescent antibody technique, indirect OR antibody formation OR serologic tests OR dna primers OR polymerase chain reaction OR dna, kinetoplast OR dna, protozoan OR Enzyme-Linked Immunospot Assay OR Electrophoresis OR Blotting, Southwestern OR Molecular Diagnostic Techniques OR Immunoassay OR Molecular Probe Techniques OR Antigens, Protozoan)

Figure 1. Terms used in PubMed search.
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variables and test results. A second researcher independently double checked the extraction of primary data from every study. Discrepancies were resolved by discussion. The following information was extracted: country in which the study was carried out, diagnostic methods applied, reference test used, characteristics of the participants, study design and quality, sample size, manufacturers and antigens used and titles for defining test positivity and outcome data (sensitivity and specificity were calculated when available data were presented). In many articles the numbers of true positive, false negative, true negative, and false positive observations were available. If not, we derived the numbers from the marginal totals and the reported sensitivity and specificity.

The number and type of participants were recorded and categorized as confirmed cases (HIV-infected individuals with VL) or controls (HIV-infected individuals without VL). Although some authors compared performance of tests in several different groups without VL, we selected only two possibilities of comparison group (control participants): 1- HIV-infected patients with the same clinical syndrome as confirmed cases with visceral leishmaniasis ruled out 2- HIV-infected patients without signs or symptoms of leishmaniasis.

Assessment of Study Quality

We assessed the quality of studies using the Quality Assessment of Studies of Diagnostic Accuracy Approach-QUADAS [21], which contains 14 items specifically developed to assess the quality of primary studies of diagnostic tests.

Data Synthesis and Statistical Analysis

The statistical analysis was based on the following steps: (1) qualitative description of findings; (2) search for the presence of publication bias, heterogeneity and threshold effect; (3) exploring possible explanations for heterogeneity; (4) statistical pooling of sensitivity, specificity and two global measures of accuracy of tests: diagnostic odds ratio (DOR) and symmetric summary receiver operating characteristic (sROC).

Publication bias was evaluated through Egger's test [22] by using Comprehensive Meta Analysis Software® v. 2.2.048 (CMA). Publication bias has been defined as the tendency on the part of investigators to submit, or the reviewers and editors, to accept manuscripts based on the direction or strength of the study findings. This definition concentrates on the fact that the strongest and most positive studies are most likely to be published.

Heterogeneity was explored with I^2 estimate from Cochran Q (the most commonly used heterogeneity statistic) according to the formula: $I^2 = 100\% \times (\text{Cochran } Q - \text{degrees of freedom})/\text{Cochran } Q$ [23]. One must understand heterogeneity as a greater variation of sensitivity, specificity or DOR between the included studies than is compatible with the play of chance. This statistical heterogeneity should represent other sources of differences such as clinical, tests or research design characteristics.

In nearly all situations sensitivity and specificity are not independent, what is called threshold effect. For this reason, sensitivity and specificity are considered inappropriate for meta-analyses, as they do not behave independently when they are pooled from various primary studies to generate separate averages [24]. The threshold effect may be caused by explicit differences in either positive cut-off definitions or implicit population and methodological differences among studies [19]. A robust approach to combining data and estimating the underlying relationship between sensitivity and specificity is the construction of a sROC curve. Methods that involve pooling sensitivities and specificities from individual studies, or combining positive and negative likelihood ratios fail to account for the paired nature of the parameters, and should generally be avoided [25].

According to Centre for Reviews and Dissemination (CRD) guidance for undertaking systematic reviews "where only one parameter (e.g. sensitivity, but not specificity) is presented, simple pooling of proportions is the only option. Assessment of single parameters is usually inappropriate, but is sometimes used when there is a specific clinical reason why only one parameter should be the focus of interest" [26]. Thus, given the small number of available studies and the paucity of data on the performance of the test in control populations (HIV-infected patients without VL), besides global analysis including few studies presented both sensitivity and specificity, we decided to pool sensitivity and specificity separately of all studies in order to compare results and check if both approaches would reach the same or different conclusions. Our intention was to discuss the methodological possibilities and assess the reliability of our results. Statistical analyses were carried out with the open source statistical language and environment R 2.0.1 [27].

To calculate sensitivity and specificity values for the tests, we cross-tabulated each result against the reference standard. Whenever possible, we extracted raw data from primary studies to fill in the four cell values of a diagnostic 2×2 table: true positives, false positives, true negatives, and false negatives. When studies did not provide confidence intervals for sensitivity or specificity, we estimated them from the reported 2×2 table [28] using Wilson score method [29].

When available, study results were pooled using a DerSimonian Laird method (random effects meta-analysis model) from Meta-Disc® 1.4 analysis software [30]. It was used to obtain pooled results of sensitivities, specificities, positive (PLR) and negative likelihood ratio (NLR). The likelihood ratio for a positive result is sensitivity divided by 1- specificity and tells how much the odds of the disease increase when a test is positive. A PLR can be used to assess the impact on diagnosis of a positive test result for an individual. The likelihood ratio for a negative result is 1- sensitivity divided by specificity and tells how much the odds of the disease decrease when a test is negative. Pooled likelihood ratio is useful since it can be used directly in the Bayes rule: Post-test odds = pre-test odds×LR. In addition, true positive rates (TPR = sensitivity) and false positive rates (FPR = 1-specificity) were summarized using a sROC curve [32]. The Q-point (point on curve where sensitivity equals specificity) obtained from the sROC curve was used as a measure of global accuracy [25]. Also used to compare overall accuracy among tests, diagnostic odds ratio (DOR) with fixed effects model were obtained from CMA® software. The DOR of a test is the ratio of PLR divided by NLR. Pooling sensitivity and specificity separately assumes that the diagnostic threshold is the same in each study. Pooling DORs relaxes this assumption by assuming that the studies relate to the same sROC curve. The DOR has been put forward as a useful single indicator of test performance, which indicates the strength of the association between test results in disease [31]. It is difficult to be clinically interpreted, but useful from the statistical point of view in the assessment of the overall test accuracy in meta-analysis [19,31,33].

Results

From the literature searches, we identified 432 primary citations from PubMed and 132 from LILACS. Seven additional articles (references from primary articles) were also found. Publication year ranged from 1989 to 2009. Study selection flow is shown in Figure 2.

All 132 citations from LILACS and 370 from PubMed were excluded by the reading of titles and/or abstracts. Thirty three

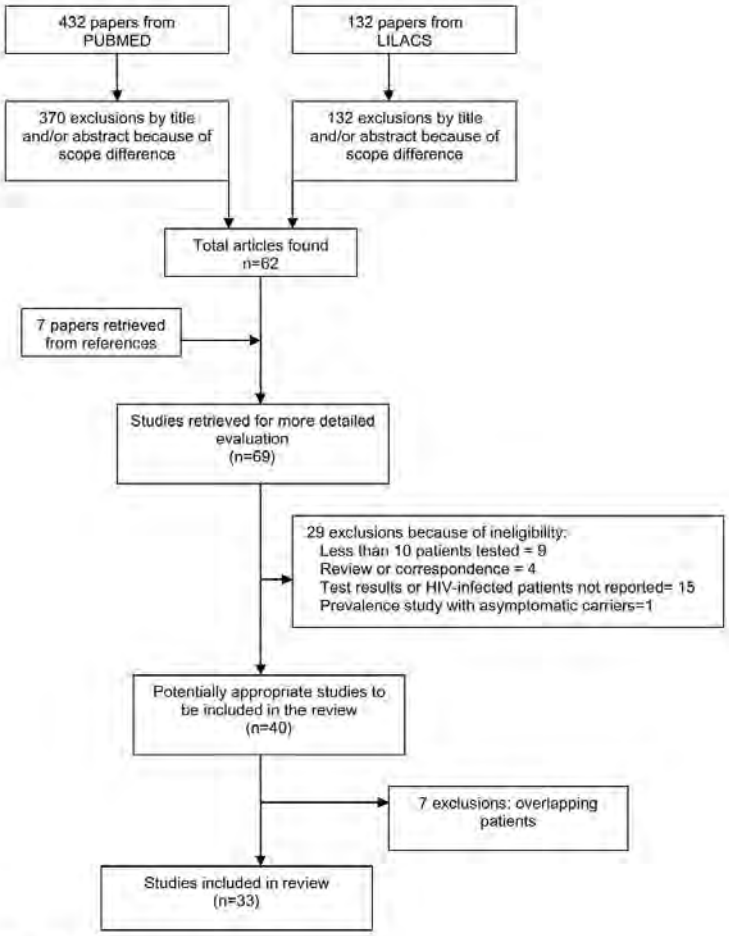


Figure 2. Study selection process.
doi:10.1371/journal.pntd.0001665.g002

more articles were excluded after reading the entire article: nine reported less than 10 patients tested, four were a review or correspondence, fifteen studies did not report tests results or the immunosupressed patients could not be identified, one was a prevalence study to detect the presence of asymptomatic carriers in a given population and seven studies were excluded because they included cases published elsewhere. Thirty three studies recruiting 1489 patients were included. A total of six different serological tests were found: direct agglutination test (DAT), indirect fluorescent antibody test (IFAT), Enzyme linked immunosorbent assay (ELISA), Immunoblotting (Blot), rapid K39-based immunochromatographic test and Haemagglutination (HA). We found only two studies [34,35] addressing the performance of two different commercial counter-immunoeletrophoresis tests (commonly referred to as rapid diagnostic tests) among HIV-infected patients. Only one from them stated sensitivity and specificity. No studies involved individuals younger than 15 years old. Table S1 summarizes the characteristics of the 33 studies.

The study quality analysis as assessed by QUADAS tool showed that 24 out of 33 studies (73%) met more than seven criteria (Table S2). Regarding study design and execution, fifteen studies were identified as retrospective or a clinical database analysis (set of data systematically gathered on all patients even though no specific analysis was prospectively planned). Sixteen reports were truly prospective and two had transversal design. In addition, a minority of them (8 studies) reported consecutive patient inclusion as the method of participant selection. Only two studies [8,36] reported at least single blinded interpretation of index test and reference standard results. For most studies information about the condition of the specimens (frozen or fresh) was unclear or not reported.

Three studies [12,37,38] reported that antibody detection was done with stored sera.

The reference standard for all studies was a positive result on direct microscopically examination or culture of blood or bone marrow aspirate, and in few cases, from another sample tissue. In one study diagnosis could also be confirmed by serology [16] or detection of parasites by polymerase chain reaction [39] associated with clinical signs. In these two studies the index test did form part of the reference standard. In 7 of 13 studies evaluating control patients, the entire study population was investigated using the identical reference standard (complete verification). In other five studies the reference standard for VL patients and control participants differed (e.g., parasitological tests for VL patients and serological tests for control participants (differential verification) and one study did not report the test used for control verification [35]. Five studies had as control group HIV-infected individuals without clinical signs of disease.

Most studies (28/33) included less than 100 patients and only 14 out 33 studies (42%) provided detailed clinical characterization of the studied population. The specific antigen composition was described in 14 out of 21 studies (62%) evaluating IFAT tests. Six from these studies used a commercial test based on axenic cultures of *L. infantum*; two other studies used commercial tests based on *L. tropica* [40] and *L. donovani* [41] culture. Seven studies used antigen prepared from whole promastigotes of the World Health Organization strain.

Published experience with ELISA is very scarce: there are six studies. Two studies searched antibodies to recombinant (r) antigen K39 while other two [18,42] used antigen extracted from promastigotas strain of *L. infantum*. The other two studies [43,44] assessing ELISA performance did not report the antigen used.

Regarding immunoblotting, most authors considered the criterion for positivity the detection of antibodies to the 14- kD antigens with or without antibodies to other low molecular weight bands [18,43 46]. Santos-Gomes and others [10] assumed as positive result the presence of any band since the sera from the control groups did not recognize any *Leishmania* antigen. Medrano et al. [8] considered an immunoblotting reactive when one or more bands of any molecular weight detected were present in at least two patients with VL, but not in the negative control sera from the no endemic area.

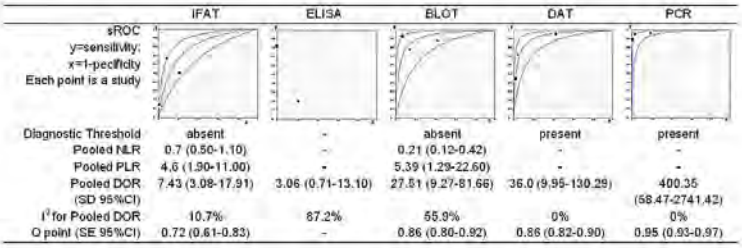
Details of the PCR techniques used are summarized in Table S1. Whole blood was used in all but four studies evaluated also PCR use in bone marrow samples [16,39,47,48]. Several variations in the PCR technique were used: small subunit ribosomal RNA (ssu-RNA) from *L. infantum* [16,39,48 50], ssu-rRNA from *L. donovani* [36], repetitive nuclear sequence (140 bp) from *L. infantum* [18] and nested-PCR (100 bp) from *L. infantum*.

Only 13 studies evaluating 5 tests [7,8,10,12,35,36,44,46,50 54] showed results of both sensitivity and specificity, the requirement to testing threshold effect presence. It should be noted however that for samples with less than 10 studies is not possible to state at significance level of 5% there is not threshold effect for studies in which the correlation result was negative.

Corresponding sROC plots of the studies and estimated DOR (95% confidence interval) of tests are shown in Figure 3. For DOR analysis, the global accuracy of DAT 36.01 (9.95 130.29, $I^2=0$) and Blot 27.51 (9.27 81.66, $I^2=56$) was comparable and higher than IFAT 7.43 (3.08 1791, $I^2=11$) and ELISA 3.06 (0.71 13.10, $I^2=87$), in spite of wide confidence intervals. PCR in whole blood had the highest DOR: 400.35 (58.47 2741.42, $I^2=0$). Pooled NLR for IFAT 0.7 (0.5 1.1) is higher than Blot 0.21 (0.12 0.42). Egger's test 2-sided p value was larger than 0.05, suggesting absence of publication bias for all tests.

Figures 4 show the results of individual and combined sensitivity and sensitivity estimates for the tests including all studies. On the whole, sensitivity varied widely among studies of a given type of test and in studies across different types of tests. There is high heterogeneity across studies for most tests (Table S3 and Table S4). Although we used random effects model to summarize data, a point estimate of separated sensitivity or specificity must be evaluated carefully. IFAT was the test most frequently evaluated in the review (21 studies) with sensitivity values ranged from 11% to 82%. Sensitivity was less than 50% in ten out 21 (48%) studies; specificity value ranged from 79% to 100%, with specificity >90% in three out of four (75%) studies. The estimated sensitivity for the IFAT using random effects model was 51% (95% confidence interval 43% to 58%). Three studies [8,40,53] had even lower sensitivity (11, 14 and 22%). Although all three had used as cutoff 1:80, we carried out separate analyses in subgroups stratified by cut-off value, sample size, study design, geographical region, and type of controls and we did not find any significant difference except for QUADAS score, which showed an inverse association with sensitivity (data not shown). It was not possible to assess the heterogeneity between studies according to geographic region due to the small number of studies outside Europe.

The estimated sensitivities using random effects model and their respective 95% confidence intervals for the other tests were: Blot 84% (75% to 91%), DAT 81% (61% to 95%), ELISA 66% (40% to 88%), PCR in whole blood 92% (83% to 98%) and PCR in



	IFAT	ELISA	BLOT	DAT	PCR
Diagnostic Threshold	absent	-	absent	present	present
Pooled NLR	0.7 (0.50-1.10)	-	0.21 (0.12-0.42)	-	-
Pooled PLR	4.6 (1.90-11.00)	-	5.39 (1.29-22.60)	-	-
Pooled DOR (SD 95%CI)	7.43 (3.08-17.91)	3.06 (0.71-13.10)	27.51 (9.27-81.66)	36.0 (9.95-130.29)	400.35 (58.47-2741.42)
I^2 for Pooled DOR	10.7%	87.2%	55.9%	0%	0%
Q point (SE 95%CI)	0.72 (0.61-0.83)	-	0.86 (0.80-0.92)	0.86 (0.82-0.90)	0.95 (0.93-0.97)

Figure 3. Tests performance summary. Footnote: Immunofluorescence antibody test (IFAT), Enzyme linked immunosorbent assay (ELISA), Immunoblotting (BLOT), direct agglutination test (DAT) and polymerase chain reaction (PCR) in whole blood, standard deviation (SD), 95% confidence interval (95% CI), *standard error (SE).* SE is a measure of precision and it is not a measure of confidence interval, which is shown in sROC plot, except for ELISA.
doi:10.1371/journal.pntd.0001665.g003

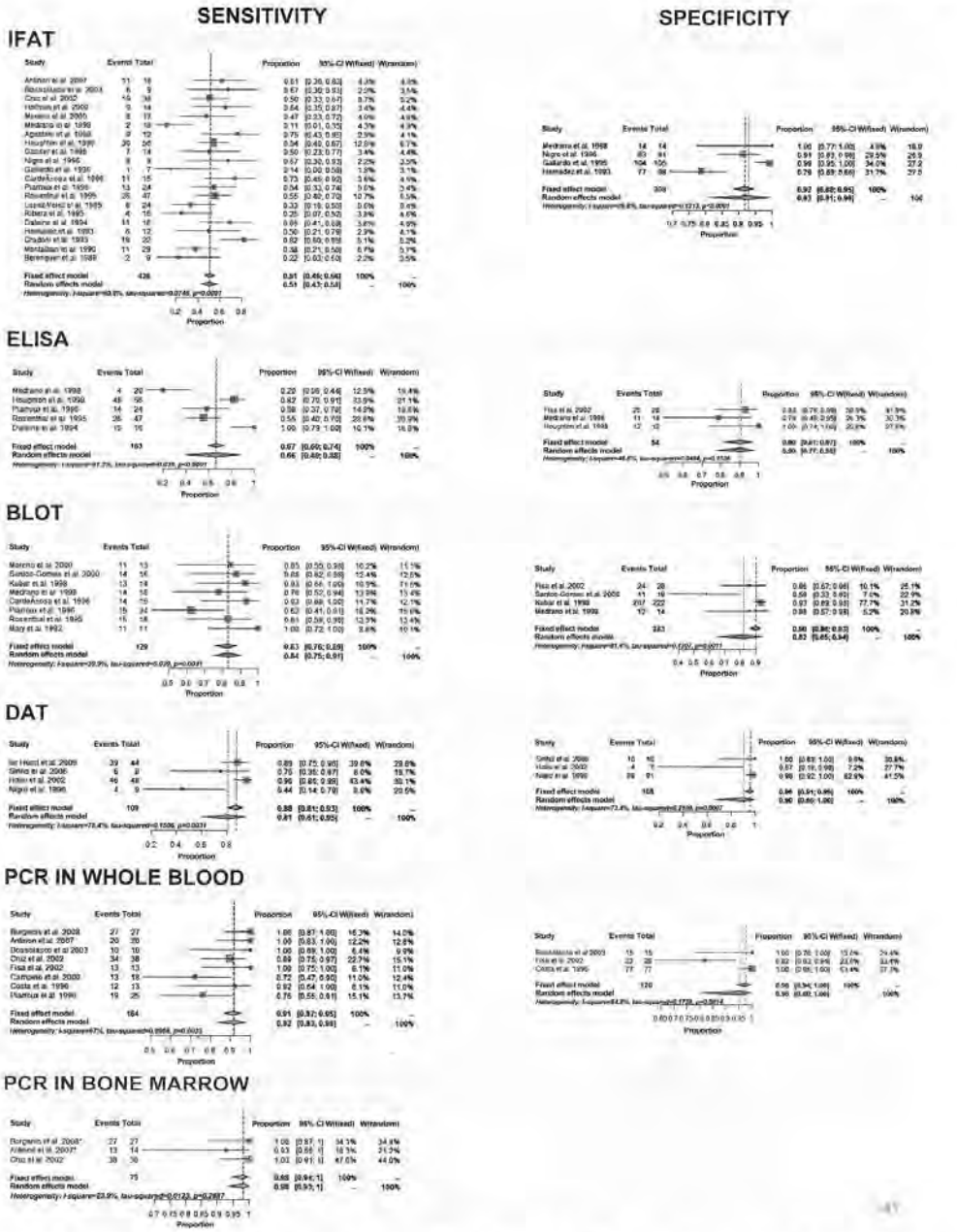


Figure 4. Estimates of sensitivity and specificity (95% confidence interval) of tests. Footnote: Combined results are shown using both options: fixed and random effects model. When both results are similar with low heterogeneity, both can be used. When they are different, we prefer results from random effects model, which gives wide and conservative confidence interval for heterogeneous results.
doi:10.1371/journal.pntd.0001665.g004

bone marrow 98% (93% to 100%). Regarding specificity, we also found significant heterogeneity for the same test across several studies but high overall pooled specificity for all of them. The estimated specificity using random effects model and their respective confidence intervals for following tests were: Blot 82% (65% to 94%), ELISA 90% (77% to 98%), IFAT 93% (81% to 99%), DAT 90% (66% to 100%), PCR in whole blood 96% (80 to 100%).

Figure 5 shows performance for PCR in peripheral blood through a sROC curve. The accuracy of PCR based on Q-point was 0.95; 95%CI 0.92 0.97, which means good overall performance.

Discussion

Our data allow some conclusions based on available evidence, which essentially reflect the European experience with serological and molecular diagnosis of VL among HIV-infected: (1) the available evidence is limited and there is great variability among the studies; (2) the accuracy of molecular methods is greater than the serological methods; (3) DAT and Blot have better global accuracy among serological tests; (4) although specificity was generally high for all serological tests, there is unexpectedly high variation in specificity among studies evaluating the same test; (5) serological tests vary widely in performance, but with overall limited sensitivity in HIV infected patients. It is very important to note that high concentration of cases of *Leishmania*-HIV co-infection is found in Africa and Asia continents and it is possible that these findings can not be extrapolated to these populations. However, this is a critical summary of the evidence currently available.

Several indicators of diagnostic performance have been proposed, such as sensitivity and specificity. Using paired indicators can be a disadvantage in comparing the performance of competing tests, especially if one test does not outperform the other on both indicators. The DOR is a single indicator of diagnostic performance; it facilitates formal meta-analysis of studies on diagnostic test performance [31]. Based on DOR, we observed superiority of PCR above serologic methods. Among serologic tests, based on DOR, we observed that Blot and DAT are superior to ELISA and IFAT. Pooled NLR (95% confidence interval) for IFAT 0.74 (0.51 1.09) is higher than Blot 0.21 (0.12 0.42), confirming that especially IFAT is not an appropriate test to exclude diagnosis, since its negative predictive value will be low. Blot and DAT had better sensitivities than the other serologic tests evaluated.

The sensitivity values of each study are consistent with the values of the studies combined and we can affirm that IFAT sensitivity is very limited and heterogeneous. An explanation for this heterogeneity is the quality of the studies, which is suggested by the indirect comparison between QUADAS score and sensitivity of IFAT. Among the studies assessing IFAT, one study [37] is distinguished by exceptionally high sensitivity displayed (81%). According to this author, the performance obtained using IFAT prepared with reference *L. infantum* strain from WHO (MHOM/TN/80/IPT1) was significantly better than prior local experience with commercial IFAT kits. This may mean that variables related to the preparation of the antigen and regional differences in prevalence and strain of *Leishmania*, in addition to the characteristics of the tested populations, have a greater impact on test performance [55].

Pooled specificity is high among all serologic tests. Nevertheless, we found great variability in the results for the same test across different studies. Especially for one DAT study [54] and one Blot

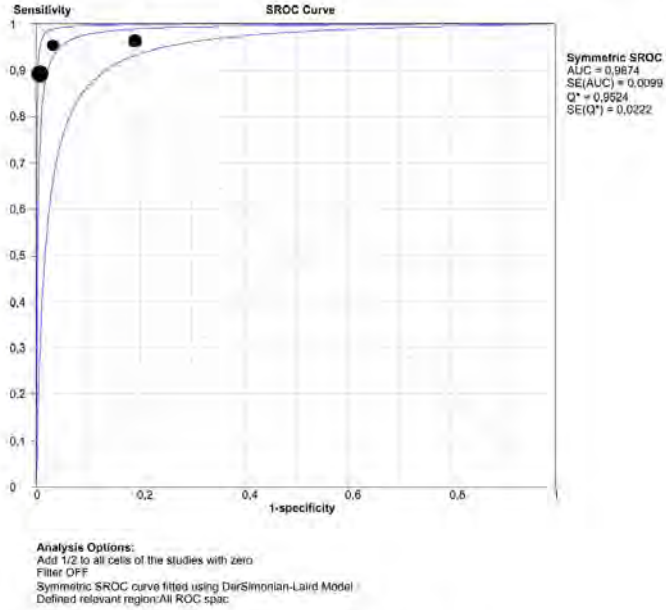


Figure 5. sROC curve for PCR in peripheral whole blood.
doi:10.1371/journal.pntd.0001665.g005

study [10], the specificity results were very low, associated with high sensitivities, suggesting threshold effect. The sensitivity and specificity of such diagnostic methods depend on the type, source, and purity of antigen employed, as some of the *Leishmania* antigens have common cross-reactive epitopes shared with other microorganisms such *Trypanosome brucei* subspecies, *Trypanosome cruzi* and *M. tuberculosis* [6]. In addition, the type of controls significantly influenced the estimates for specificity. Studies including healthy controls tend to show higher specificity than those recruiting patients with clinically suspected disease consecutively and prospectively in a representative clinical setting.

DAT based on whole promastigotes of *L. donovani* or *L. infantum* are tests used widely for the diagnosis of VL. However, the major disadvantage of this technique is the limited production facility of quality controlled antigen. A recent meta-analysis of the DAT performance among immunocompetent individuals showed sensitivity and specificity estimates (and 95% confidence interval) of 94.8% (92.7% to 96.4%) and 97.1% (93.9% to 98.7%), respectively [56]. Despite lower performance in HIV-infected patients than in immuncompententes, DAT (and Blot) proved to be the most effective serological technique in those immunosupressed by HIV infection. However, only four studies assessing DAT with sensitivities and specificities, none from Latin-America and only one from Europe could be included. One [7] out of these four studies exhibited discrepant and very low sensitivity, despite use of 1:400 cut-off. Specifically this study was performed in Italy (the others were conducted in India and Ethiopia) and DAT was carried out using promastigotes of *L. donovani sensu latu*. This may represent the relevance of the prevalent strain in the performance of a test prepared from promastigotes (local antigen specificity) or the difference in immune response induced by more or less anthroponotic strains of *Leishmania*.

Some heterogeneity in sensitivity of the tests seemed to be related to the geographical location of the study. Differences in test performance between regions is attributable basically to parasite diversity [57], but it can also be related to differences in antibody concentrations which may in turn be linked to different age patterns, immune and/or nutritional status of patient. In this review, it was not possible to evaluate the test's performances in various endemic regions of world. All included studies assessing IFAT, Blot and ELISA were performed in Europe. Data on DAT essentially reflect the response in Ethiopia and Italy, with only one study performed in India (few patients). No study from the Americas was found. Regarding rK39 dipstick test, the only two studies [34,35] found, one carried out in India and one in Ethiopia, exhibited different sensitivities. Similarly, among non HIV-infected patients results between global regions were substantially different. There is data showing the low sensitivity of rK39 based dipsticks in Sudan [58 60] and better results in studies from South Asia [56].

Serological tests have an already recognized low sensitivity for the diagnosis of VL among HIV-patients [61]. Gradoni et al. [37] suggested that the serological response could be related to the sequence of temporal acquisition of the infectious agents. Seropositivity would represent a reactivation of latent infection before the immune depression caused by the viral infection (asymptomatic carriers), while seronegativity would result from primary *Leishmania* infections after viral infection. However, the severe dysfunction of T and B lymphocytes in HIV-infected individuals, an alteration in antigen presentation by macrophages or in T and B lymphocyte cooperation would explain the decrease in specific antibody production, as occurs for other infections [1]. It is also necessary to note that most serological studies from Europe date from the early stages of the HIV epidemic, while the PCR studies were usually done when HAART was available. Possibly, different types of populations (more advanced HIV disease in the earlier studies) were included.

Standard techniques for assessing diagnostic tests assume that a definitive reference test is available, that is, that the reference test used is as close to 100% accurate as can be. However, it may be either that the available test is far from perfect or that such a test simply does not exist [23]. The presence of *Leishmania* parasites may only be demonstrated incontrovertibly by the microscopically examination of smears or the culture of blood or biopsy samples. Microscopical examination of spleen aspirates is sensitive and specific but requires expertise to carry out the aspiration safely and to read the slides accurately. Examination of bone marrow or lymph node aspirates is equally specific but less sensitive [62]. Parasite load is quite heavy in VL-HIV co-infected patients and the presence of *Leishmania* amastigotes in the bone marrow can often be demonstrated. However there are well-described instances in the literature where amastigotes were not demonstrable in bone marrow, though they were found at unexpected locations like the stomach, the colon, or the lungs [5]. The majority (31/33, 94%) of studies used exclusively microscopic determination of parasites as the reference standard. Although direct and culture are considered the definitive diagnosis, parasitological methods does not detect all cases of VL; therefore, some degree of misclassification of disease for study participants was possible.

In the case of molecular tests, previous studies suggest their greater sensitivity compared to the classical parasitological methods [13]. As a result, some authors used a combination of several laboratory tests and clinical manifestations as reference test. Incorporation bias occurs where the experimental test is used as part of the reference strategy, that is, the experimental test and reference tests are not independent, leading to overestimation of both sensitivity and specificity. Based on prevalence studies, the proportion of individuals identified as asymptomatic carriers of *Leishmania* by PCR methods is not negligible [63]. None of the studies testing PCR included here assessed the proportion of asymptomatic patients co-infected with *Leishmania* and HIV. An important point to notice is that molecular tests are still expensive and require sophisticated laboratory setting; these features represent real obstacles to their implementation in the regions with the highest absolute numbers of HIV-VL coinfection cases (East-Africa and India). This performance data can be used to guide priority setting for field trials and/or procurement decisions. The final decision on product selection needs to be taken in a rational way, considering not only the minimal performance limits, but also the global endemic region, patient characteristics, experience of the intended users, climate and costs.

During the past few years, numerous studies have investigated *Leishmania* antigen expressions at the level of specific antibody recognition. Using immunoblotting techniques, several *L. infantum* antigens that appeared promising for establishing an immunodiagnosis of VL in nonimmunocompromised hosts have been identified [8]: 70 72 kD, 94 kD, 14 16 kD, 39 kD, 24 and 32 kD, but a clear pattern of specific immune response to parasite antigens during the active course of the disease has not been yet defined. Mary and others [64] in a series of 11 AIDS cases found a similar pattern of reactivity between HIV and non-HIV patients with VL that differed only in the variable presence of a 14-kD band in the former group. The 16-kD antigenic component was considered as the more sensitive and specific diagnostic band. Rosenthal and others [43] in another study carried out in the same endemic area (southern France) reported the presence of bands of molecular mass 14 kD and 16 kD in 15 of the 18 evaluated cases. In Medrano et al [8] study, immunoblots were found to be reactive

during the active course of the disease in 78% of the cases. Five groups of parasite antigens (14 kD, 42–43 kD, 57 kD, 76 kD, and 94 kD) appear to have potential use for diagnosis although the pattern of reactivity observed during the acute VL disease was very variable. Among the nine studies evaluating immunoblotting, performance described by Piarroux and colleagues [18] is distinguished by low sensitivity (63%). In this study, unlike the others, a more sensitive reference test was used and included visualization of *Leishmania* in any specimen collected at the same period, besides in bone marrow aspirate. However, strict comparisons between results reported in the literature are rather difficult because of the variability in the techniques and the use of different strains and antigens. Considering the high variability of the immunoblotting patterns, it seems that a combination of several antigens should be used, as has been previously suggested [8,65].

Moreover, different settings (e.g., difference in *Leishmania* prevalence may have accounted for some variation in test performance. It is often assumed that indices of test accuracy such as sensitivity and specificity are fixed (for a given threshold). But they can vary as a function of prevalence [66,67]. When spectrum bias is present, either sensitivity or specificity would be expected to change. Sensitivity would be expected to increase where test results become more extreme in patients with the most severe disease (i.e. more likely to test positive). Specificity is affected by a variety of alternative diagnosis in those without the target disorder that could cause false positive results. The range of such diagnosis is likely to be wider in studies that have a lower prevalence of the target disorder [23]. Another problem concerned to limited information on clinical status and disease severity in the populations tested. Differing criteria for patient selection, age, duration of illness and severity of HIV-disease of the study populations may have introduced significant variability in findings among studies (selection bias).

There are also limitations in studies methods. The differences in PCR methods included the nature of the samples (whole blood or bone marrow aspirate), volume tested, DNA extraction procedures, choice of target gene, detection of PCR products and the use of appropriate controls. All of these factors have been reported as likely causes of heterogeneity and they were all present in the studies included. Interpretation of many diagnostic tests involves some degree of subjective interpretation. Only two of the studies (6%) reported blinded interpretation of the results of the index test and the reference standard. Lack of blinding may have resulted in an overestimation of the sensitivity of the index test result. In additional, the condition of specimens may also have affected the sensitivity results. The vast majority (91%) of studies did not report if frozen or fresh sera were used. In 5 from 13 studies (38.5%), different diagnostic tests were performed in VL patients and control participants: parasitological tests for patients and serological test for control participants [verification bias]. In one study [35] the information about the test used to rule out VL was not reported.

This comprehensive review is mainly limited by quality of available studies. We believe that pooled measures from different studies help to appraise global accuracy. Nevertheless, its validity remains on scarce evidence that may change as larger well designed studies are done. No large prospective clinical studies evaluating either serological or molecular tests have been reported. In addition, available data are not representative of all endemic regions. Data about HA, rapid K39-based immunochromatographic and PCR test in bone marrow aspirate could not be analyzed due to the paucity of studies. The same way, there were too few studies to explore by subgroup analysis or metaregressing whether the diagnostic yield of the methods was different among subgroups (i.e., control characteristics, sample size, study design and quality) or whether the different techniques influenced the results. The heterogeneity among studies evaluating the same method makes pooled sensitivity and specificity measures less reliable. The strategies of pooling sensitivity and specificity or using global accuracy measures like DOR and sROC are subject to different kind of bias. Including studies with more quality, although in low number, reduce the bias of methodological flaws changing results. Including more studies pooling separately sensitivity and specificity reduce the bias of selecting low number of studies. In spite of these different biases, by using both methods we found very similar results confirming the consistency of these observations.

Conclusions

The results of this evaluation confirm the low sensitivity of the serologic tests for VL diagnosis in HIV-infected patients. Except for DAT, currently available evidence about performance of serological tests refers to *Leishmania infantum chagasi*, the etiological agent of visceral leishmaniasis in the Americas and in the Mediterranean basin. Our results indicated superiority of Blot over IFAT and ELISA. DAT seems to be better than IFAT and ELISA, like Blot, but its performance may be influenced by difference in geographic region, meaning different *Leishmania* species. As the performance of DAT and Blot is comparable, the choice should be made on the basis of other criteria such as region, cost, feasibility, and sustainability. Given these findings, we express concern that IFAT remains the most frequently serological test used for VL investigation in South America, even among HIV-infected. At this time, there is no evidence to support recommendations on serologic or molecular diagnosis of VL in patients infected with HIV and living in East Africa or Southeast Asia. The development of the rK39 dipstick has brought a major improvement in the diagnosis of VL in non HIV-infected patients in the field. Nevertheless, the paucity of data about the rK39 dipstick in HIV-infected patients underscores the need for more research before it being integrated in a diagnostic algorithm.

In spike of lack of homogeneity of the PCR methods used, available evidence suggests that, at this point in time, published data on molecular tests produce consistently good estimates of accuracy. Its main weaknesses are the lack of standardization for the technique. We must also point out that the meaning of *Leishmania* infection detected by PCR in asymptomatic individuals is not yet defined. This fact might raise questions about possible false-positive results. In addition, alternative methods must be developed to solve the "gold standard problem". A promising strategy is Bayesian latent class models [68].

More studies are needed to compare tests for VL diagnosis in different regions. This highlights the need to implement a diagnostic algorithm as appropriate for each global endemic area. The design of diagnostic studies must follow the STARD initiative [69] as a way to minimize bias.

In conclusion, based on the available evidence, serology should not be used to rule out a diagnosis of VL among HIV-infected patients. An additional molecular or parasitological test may be necessary if results of serological tests are negative. A positive serological test at even low titers has diagnostic value when combined with the clinical case definition. In its turn, tests based on PCR are highly sensitive and should contribute to the diagnosis, especially in areas of low endemicity.

Supporting Information

Table S1 The characteristics of studies. Footnote: DiaMed IT-Leish (DiaMed AG, Switzerland) ‡ Kalazar Detect Rapid

Test (In Bios International, Inc., Seattle, USA) PB: peripheral blood BMA: bone marrow aspirate bp: base pair n-PCR: nested PCR ssU-rRNA: small subunit ribosomal RNA NR: not reported NA: not applicable PCR: polymerase chain reaction # only new visceral leishmaniasis cases included, number of patients with relapses not reported.
(DOC)

Table S2 QUADAS scoring for each study. Footnote: If the answer is "no" or "unclear" = score 0. If the answer is "yes" (x) = score 1. QUADAS ITENS: 1. Was the spectrum of patients representative of the patients who will receive the test in practice? 2. Were selection criteria clearly described? 3. Is the reference standard likely to correctly classify the target condition? 4. Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests? 5. Did the whole sample or a random selection of the sample, receive verification using a reference standard of diagnosis? 6. Did patients receive the same reference standard regardless of the index test result? 7. Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)? 8. Was the execution of the index test described in sufficient detail to permit replication of the test? 9. Was the execution of the reference standard described in sufficient detail to permit its replication? 10. Were the index test results interpreted without knowledge of the results of the reference standard? 11. Were the reference standard results interpreted without knowledge of the results of the index test? 12. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice? 13. Were uninterpretable/intermediate test results reported? 14. Were withdrawals from the study explained?
(DOC)

Table S3 Individual performance of studies evaluating serological tests.
(DOC)

Table S4 Individual performance of studies evaluating molecular tests. Footnote: polimerase chain reaction (PCR).
(DOC)

Checklist S1 PRISMA checklist. Footnote: *From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097.
(DOC)

Author Contributions

Conceived and designed the experiments: GFC MRS AR. Performed the experiments: GFC MRS. Analyzed the data: GFC MRS FND. Wrote the paper: GFC MRS.

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Table 1. The characteristics of studies

Reference	Country	Test index IFAT and DAT (cut-off) ELISA (antigen) molecular test (type of PCR) Blot (bands used as positive criteria)	Sample size (VL-HIV /controls)	Study design	Reference test	Quadas score	Controls (HIV-Infected without VL)
ter Horst et al 2009	Ethiopia	DAT (1:3200) K39rapid test: DiaMed-IT-Leish ® †	44/-	Prospective, comparative of tests	Parasitological	9	NA
Borgeois et al 2008	France	PCR (PB and BMA): ssU-rRNA (603 bp): *L. infantum*	27/-	Prospective	Parasitological and/or PCR	7	NA
Antinori et al. 2007	Italy	IFAT (1:40) PCR (PB and BMA): ssU-rRNA (359 bp): *L. infantum*	20/-	Prospective, comparative of tests	Parasitological and/or serologic test	7	NA
Goswami et al.2007	India	K39rapid test: Kalazar Detect Rapid Test® ‡	12/60	Prospective	Parasitological	7	Symptomatic HIV-infected without VL confirmation
Sinha et al. 2006	India	DAT (1:800)	8/10	Prospective	Parasitological	6	Asymptomatic HIV-infected patients
Bossolasco et al. 2003	Italy	IFAT (1:80) PCR (PB): ssU-rRNA real-time:*L. infantum*	10/15	Prospective	Parasitological	10	Symptomatic HIV-infected without VL confirmation
Cruz et al. 2002	Spain	IFAT (1:80) PCR (PB and BMA): ssU-rRNA n-PCR (358 bp): *L. infantum*	38/-	Prospective	Parasitological	12	NA
Fisa et al. 2002	Spain	ELISA (NR) BLOT (bands 70, 65, 46, 30, 28, 14 or 12 kD) PCR (PB): n-PCR (100 bp): *L. infantum*	15/28	Prospective	Parasitological	10	Symptomatic HIV-infected without VL confirmation
Hailu et al. 2002	Ethiopia	DAT (1600)	51/7	Retrospective	Parasitological	11	Symptomatic HIV-infected without VL confirmation
Campino et al. 2000	Portugal	PCR (PB): ssU-rRNA (600 bp): *L. infantum*	18/-	Retrospective	Parasitological	8	NA
Moreno et al. 2000	Spain	IFAT (1:80) BLOT (any band)	17/-	Retrospective	Parasitological	10	NA
Hofman et al. 2000	France	IFAT (1:80)	16/-	Retrospective	Parasitological	11	NA
Santos-Gomes et al. 2000	Portugal	BLOT (at least one band)	16/-	Prospective, comparative tests	Parasitological	9	Symptomatic HIV-infected without VL confirmation
Medrano et al. 1998	Spain	IFAT (1:80) ELISA (K39 antigen) BLOT (any band)	20/14	Retrospective	Parasitological	11	HIV-1-infected patients who died of a non-VL cause
Houghton et al. 1998	Italy	IFAT (1:80) ELISA (K39 antigen)	56/12	Transversal, comparative	Parasitological	9	Asymptomatic HIV-infected patients
Kubar et al. 1998	France	BLOT (bands 14 or 18 kD)	14/222	Prospective	Parasitological	9	Asymptomatic HIV-infected patients
Agostoni et al. 1998	Italy	IFAT (1:40)	22/-	Retrospective	Parasitological	12	NA
Costa et al. 1996	France	PCR (PB): ssu-Rrna (nr): *L. Donovani*	13/77	Prospective	Parasitological	7	Symptomatic HIV-infected without VL confirmation
Gasser et al. 1996	Spain	IFAT (1:40)	19/-	Retrospective	Parasitological	8	NA
Nigro et al. 1996	Italy	IFAT (1:100) DAT (1:400)	9/91	Prospective	Parasitological	8	Asymptomatic HIV-infected patients
Piarroux et al. 1996 #	Spain	IFAT (1:80) ELISA (promastigotes *L. infantum MCAN/FR/73/LPM 56)* Blot (bands 14 or 16 kD) PCR (PB): Repetitive nuclear sequence (140 bp): *L. infantum*	25/-	Prospective	Parasitological	8	NR
Gallardo et al. 1996	Spain	IFAT (1:80)	7/105	Transversal	Parasitological	10	Asymptomatic HIV-infected patients
Cardeñosa et al. 1996	Spain	IFAT (1:80) BLOT	15/-	Retrospective	Parasitological	6	NA
Rosenthal et al. 1995	France	IFAT (NR) ELISA (NR) BLOT (bands 14 or 16 kD)	50/-	Retrospective	Parasitological and/or serologic test	11	NA
Ribera et al. 1995	Spain	IFAT (1:40)	20/-	Retrospective	Parasitological	12	NA
López-Velez et al. 1995	Spain	IFAT (1:80)	25/-	Retrospective	Parasitological	10	NA
Daleine et al. 1994	France	IFAT (1:100) ELISA (promastigotes *L. infantum MON- 1 Biokema)*	16/-	Retrospective	NR	3	NA
Hernandez et al. 1993	Spain	IFAT (1:40)	19/103	Prospective	Parasitological	6	Symptomatic HIV-infected without VL confirmation
Gradoni et al. 1993	Italy	IFAT (1:80)	22/-	Retrospective	Parasitological	6	NA
Mary et al. 1992	France	BLOT (bands 14 or 16 kD)	11/-	Prospective	Parasitological	10	NA
del Mar et al. 1991	Spain	HA (1:60)	12/-	Prospective	Parasitological	9	NA
Montalban et al. 1990	Spain	IFAT (1:40) HA	40/-	Retrospective	Parasitological	8	NA
Berenguer et al. 1989	Spain	IFAT (1:80)	9/-	Retrospective	Parasitological	10	NA

DiaMed IT-Leish (DiaMed AG, Switzerland) ‡ Kalazar Detect Rapid Test (In Bios International, Inc., Seattle, USA) PB: peripheral blood BMA: bone marrow aspirate bp: base pair n-PCR: nested PCR ssU-rRNA: small subunit ribosomal RNA NR: not reported NA: not applicable PCR: polymerase chain reaction # only new visceral leishmaniasis cases included, number of patients with relapses not reported

Table 2. QUADAS scoring for each study

QUADAS item Reference	1	2	3	4	5	6	7	8	9	10	11	12	13	14	*FINAL QUADAS SCORE*
ter Horst et al. 2009	X	x		x				x	x	x	x		x	x	9
Borgeois et al 2008	X	x		x				x	x			x	x		7
Antinori et al. 2007	X	x	x	x				x	x			x			7
Goswami et al. 2007	X		x	x	x		x	x	x						7
Sinha et al. 2006	X		x	x			x	x	x						6
Bossolasco et al. 2003	X	x	x	x	x	x	x					x	x	x	10
Cruz et al. 2002	X	x	x	x	x	x	x	x	x			x	x	x	12
Fisa et al. 2002	X	x	x	x	x	x		x	x			x	x		10
Hailu et al 2002	X	x	x	x	x			x	x	x		x	x	x	11
Campino et al. 2000	X	x	x	x	x	x		x	x						8
Moreno et al. 2000	X	x	x	x	x	x	x	x	x				x		10
Hofman et al. 2000	X	x	x	x	x	x	x	x	x				x	x	11
Santos-Gomes et al. 2000	X	x	x	x	x			x	x					x	9
Medrano et al. 1998	X		x	x	x	x	x	x	x			x	x	x	11
Houghton et al. 1998	X		x	x	x	x	x	x	x				x		9
Kubar et al. 1998	X	x	x	x			x	x	x				x	x	9
Agostoni et al.1998	X	x	x	x	x	x	x	x	x			x	x	x	12
Costa et al. 1996	X		x					x		x	x		x	x	7
Gasser et al. 1996	X		x	x	x			x	x	x	x				8
Nigro et al. 1996	X	x	x	x				x	x				x	x	8
Piarroux et al. 1996	X	x	x	x				x	x				x	x	8
Gallardo et al. 1996	X	x	x	x	x	x	x	x					x	x	10
Cardeñosa et al. 1996	X		x		x		x	x	x						6
Rosenthal et al. 1995	X	x	x	x	x	x	x	x	x				x	x	11
Ribera et al. 1995	X	x	x	x	x	x	x	x	x			x	x	x	12
López-Velez et al. 1995	X	x	x	x	x	x	x	x	x				x		10
Daleine et al. 1994	X						x	x							3
Hernandez et al. 1993	X		x	x			x	x	x						6
Gradoni et al. 1993	X		x	x	x		x	x							6
Mary et al. 1992	X	x	x	x	x	x	x	x	x			x			10
del Mar et al. 1991	X	x	x	x	x	x	x	x	x						9
Montalban et al. 1990	X	x	x	x	x			x	x				x		8
Berenguer et al. 1989	X	x	x	x	x	x	x	x	x				x		10

If the answer is "no" or "unclear" = score 0 If the answer is "yes" (x) = score 1 QUADAS ITENS: 1. Was the spectrum of patients representative of the patients who will receive the test in practice? 2. Were selection criteria clearly described? 3. Is the reference standard likely to correctly classify the target condition? 4. Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests? 5. Did the whole sample or a random selection of the sample, receive verification using a reference standard of diagnosis? 6. Did patients receive the same reference standard regardless of the index test result? 7. Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)? 8. Was the execution of the index test described in sufficient detail to permit replication of the test? 9. Was the execution of the reference standard described in sufficient detail to permit its replication? 10. Were the index test results interpreted without knowledge of the results of the reference standard? 11. Were the reference standard results interpreted without knowledge of the results of the index test? 12. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice? 13. Were uninterpretable/ intermediate test results reported? 14. Were withdrawals from the study explained?

Table 3. Individual performance of studies evaluating serological tests

Reference	Country	True positive	False positive	False Negative	True negative	Sensitivity 95% Confidence interval	Specificity 95% Confidence interval
IFAT							
Antinori et al. 2007	Italy	11		7		0.611 0.386-0.797	
Bossolasco et al. 2003	Italy	6		3		0.667 0.354-0.879	
Cruz et al. 2002	Spain	19		19		0,500 0.348-0.651	
Moreno et al. 2000	Spain	8		9		0.471 0.262-0.690	
Hofman et al. 2000	France	9		5		0.643 0.388-0837	
Medrano et al. 1998	Spain	2	0	16	14	0.111 0,031-0,328	1 0.785-1.000
Agostoni et al. 1998	Italy	9		3		0.750 0.468-0.911	
Houghton et al. 1998	Italy	30		26		0.536 0.407-0.659	
Gasser et al. 1996	Spain	7		7		0.500 0.268-0.732	
Nigro et al. 1996	Italy	6	8	3	83	0.667 0.354-0.879	0.912 0.836-0.955
Gallardo et al. 1996	Spain	1	1	6	104	0.143 0.007- 0.513	0.990 0.948-0.995
Cardeñosa et al. 1996	Spain	11		4		0.733 0.480-0.891	
Piarroux et al. 1996	Spain	13		11		0.541 0.351-0.721	
Rosenthal et al. 1995	France	26		21		0.553 0.412-0.686	
López-Velez et al. 1995	Spain	8		16		0.333 0.179-0.533	
Ribera et al. 1995	Spain	4		12		0.250 0.102-0.495	
Daleine et al. 1994	France	11		5		0.687 0.444-0.858	
Hernadez et al. 1993	Spain	6	21	6	77	0,500 0.254-0.746	0.786 0.694-0.855
Gradoni et al. 1993	Italy	18		4		0.818 0.615-0.927	
Montalban et al. 1990	Spain	11		18		0.379 0.227-0.56	
Berenguer et al. 1989	Spain	2		7		0.222 0.063-0.547	
ELISA							
Fisa et al. 2002	Spain		2		26		0.928 0.773-0.980
Medrano et al. 1998	Spain	4	3	16	11	0.200 0.081-0.416	0.786 0.524-0.924
Houghton et al. 1998	Italy	46	0	10	12	0.821 0.702-0.900	1 0.758-1.000
Piarroux et al. 1996	Spain	14		10		0.583 0.388-0.755	
Rosenthal et al. 1995	France	26		21		0,553 0.412-0.686	
Daleine et al. 1994	France	16		0		1 0.806-1	
BLOT							
Fisa et al. 2002	Spain		4		24		0.857 0.685-0.943
Moreno et al. 2000	Spain	11		2		0.846 0.578-0.957	
Santos-Gomes et al. 2000	Portugal	14	8	2	11	0.875 0.639-0.965	0.578 0.363-0.769
Kubar et al. 1998	France	13	15	1	207	0.928 0.685-0.996	0.932 0.892-0.959
Medrano et al. 1998	Spain	14	2	4	12	0.778 0.548-0.91	0.857 0.600-0.959
Cardeñosa et al. 1996	Spain	14		1		0.933 0.702-0.997	
Piarroux et al. 1996	Spain	15		9		0,625 0.427-0.788	
Rosenthal et al. 1995	France	15		3		0.833 0.608-0.942	
Mary et al. 1992	France	11		0		1 0.741-1	
DAT							
ter Horst et al. 2009	Ethiopia	39		5		0.886 0.760-0.950	
Sinha et al. 2006	India	6	0	2	10	0,750 0.409-0.928	1 0.722-1.000
Hailu et al. 2002	Ethiopia	46	3	2	4	0.958 0.860-0.988	0.571 0.250- 0.842
Nigro et al. 1996	Italy	4	2	5	89	0.444 0.189-0.733	0.978 0.923-0.994
RECOMBINANT K39 DIPSTICK TEST (IMMUNOCHROMATOGRAPHIC TEST)							
ter Horst et al. 2009	Ethiopia	34		10		0.773 0.630-0.872	
Goswami et al. 2007	India	12	0	0	60	1 0.757-1	1 0.939-1

Table 4. Individual performance of studies evaluating molecular tests

Reference	Country	True positive	False positive	False Negative	True negative	Sensitivity 95% Confidence interval	Specificity 95% Confidence interval
PCR (BONE MARROW)							
Borgeois et al. 2008*	France	27		0		1 0.875-1	
Antinori et al. 2007*	Italy	13		1		0,928 0.685-0.996	
Cruz et al. 2002	Spain	38		0		1 0.908-1	
PCR (PERIPHERAL BLOOD)							
Borgeois et al. 2008	France	27		0		1 0.875-1	
Antinori et al. 2007	Italy	20		0		1 0.839-1	
Bossolasco et al 2003	Italy	10	0	0	15	1 0.722-1.000	1 0.796-1.000
Cruz et al. 2002	Spain	34		4		0,895 0.759-0.958	
Fisa et al. 2002	Spain	13	5	0	23	1 0.772-1.000	0.821 0.644-0.921
Campino et al. 2000	Portugal	13		5		0,722222 0.491-0.875	
Costa et al. 1996	France	12	0	1	77	0.923 0.667-0.996	1 0.952-1.000
Piarroux et al. 1996	Spain	19		6		0,76 0.566-0.885	

polimerase chain reaction (PCR)

ARTIGO 3

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"Efficacy of anti-Leishmania therapy in visceral leishmaniasis among HIV infected patients: a systematic review with indirect comparison"

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Efficacy of Anti-Leishmania Therapy in Visceral Leishmaniasis among HIV Infected Patients: A Systematic Review with Indirect Comparison

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Abstract

Objective: We conducted a systematic literature review with indirect comparison of studies evaluating therapeutic efficacy and toxicity associated to visceral leishmaniasis (VL) therapy among HIV infected individuals.

Main outcome measurements: The outcomes of interest were clinical and parasitological cure, mortality, and adverse events.

Methods: PRISMA guidelines for systematic reviews and Cochrane manual were followed. Sources were MEDLINE, LILACS, EMBASE, Web of Knowledge databases and manual search of references from evaluated studies. We included all studies reporting outcomes after VL treatment, regardless of their design. Study quality was evaluated systematically by using the Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. Comprehensive Meta-Analysis software v.2.2.048 was used to perform one-group meta-analysis of study arms with the same drug to estimate global rates of success and adverse events with each drug. These estimates were used, when possible, to indirectly compare treatment options, adjusted for CD4 count. Direct comparison was pooled when available.

Results: Seventeen studies reporting five treatment regimens and outcome of 920 VL episodes occurring in HIV infected individuals were included. The main outstanding difference in outcome among the treatment regimens was observed in mortality rate: it was around 3 times higher with high-dose antimony use (18.4%, CI 95% 13.3–25%), indirectly compared to lipid formulations of amphotericin B treatment (6.1%, CI 95% 3.9–9.4%). It was observed, also by indirect comparison, higher rates of clinical improvement in study arms using amphotericin B than in study arms using pentavalent antimonial therapy (Sb^V). The parasitological cure, an outcome that presented some degree of risk of selection and verification bias, had rates that varied widely within the same treatment arm, with high heterogeneity, hampering any formal comparison among drugs. One direct comparison of amphotericin and antimoniate was possible combining results of two studies and confirming the superiority of amphotericin.

Conclusions: Available evidence suggests that amphotericin is superior to antimony treatment. Death rate using antimoniate high dose is unacceptably high. Randomized controlled trials are necessary to compare different formulations and doses of amphotericin, alternative therapies and drug combinations.

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Introduction

In recent years, several reports have emphasized the increasing importance of visceral leishmaniasis (VL) as an opportunistic infection among HIV-positive patients in areas where both infections are endemic [1]. Chemotherapeutic agents with efficacy against VL include amphotericin B, pentavalent antimonial drugs, paramomycin (a parenteral aminoglycoside) and miltefosine (the first oral drug for treatment of VL). The pentavalent antimonial drugs (Sb^V), sodium stibogluconate (SSG) and meglumine antimoniate have been used for past decades as the first line drugs for treatment because of their low cost and availability in most countries. Currently this option has been sidelined since more efficacious and less toxic alternatives exist [2]. Amphotericin B deoxycholate has high antileishmanial efficacy but it is associated with high risk of renal toxicity and other side effects and has been replaced in recent years in countries with sufficient financial resources by lipidic formulations of the drug. Pentamidine isethionate, a second-line alternative treatment is rarely used due to suboptimal efficacy and toxicity [3]. Although VL is treated

Author Summary

In co-infection with HIV/AIDS, visceral leishmaniasis (VL) most often results in an unfavorable response to treatment, frequent relapses, and in premature deaths. Scarce data is available regarding the treatment of leishmaniasis in HIV-infected patients (VL-HIV). Despite this, clinical decisions must be made. To aid in this task we reviewed comprehensive and systematically the available literature about efficacy and toxicity of therapeutic options for VL-HIV. PRISMA guidelines and Cochrane manual for systematic reviews were followed. Direct and indirect comparisons of nonrandomized studies were used, adjusting for CD4 count. Seventeen studies reporting five treatment regimens and outcome of 920 VL episodes occurring in HIV infected individuals were included. Results suggest higher survival and clinical response rate with amphotericin B than with antimony treatment. Antimonial therapy carries a higher rate of drug discontinuation and a significantly higher mortality indirectly compared to treatment with amphotericin B. Randomized controlled trials are needed to compare doses and formulations of amphotericin and alternative treatments.

similarly in patients with and without HIV infection, co-infected patients generally have low cure and high mortality rates [4,5]. Furthermore, HIV-infected patients are more likely to suffer treatment-related adverse events than the HIV-negative population [6,7].

Despite the prevalence, clinical implications and epidemiological impact of *Leishmania*/HIV co-infection, surprisingly scarce data is available regarding the treatment of leishmaniasis in HIV-infected patients. Major challenges include widespread resistance to pentavalent antimonial compounds, high treatment failure, toxicity and relapse rates [8]. The optimal therapy, including duration and dosages remain to be established. Indirect comparisons of nonrandomized studies are useful in the absence of randomized controlled trials, knowing its limitations and its assumptions [9,10], adjusting for important covariates such as CD4 lymphocyte count. The aim of this study is to perform a systematic literature review regarding therapeutic efficacy and toxicity associated with visceral leishmaniasis therapy among HIV infected individuals, making comparisons of treatment options when possible.

Methods

The review methodology followed the recommendations published by PRISMA guidelines [11] for systematic reviews and Cochrane Collaboration Group [12]. The search was done until 30, September 2012. A literature search was performed in PubMed, EMBASE, LILACS and WEB OF KNOWLEDGE databases using the search terms 'visceral leishmaniasis', 'HIV infections', 'therapy', without language or publication status restrictions.

Studies were included if reporting response to therapy to VL occurring in individuals with HIV infection. Literature search was not limited to randomized controlled trials, including all study designs. Studies involving less than ten patients; or containing a mixed population where data from HIV-infected patients or from different treatment arms could not be extracted separately were excluded. Study selection was made independently by two reviewers (GFC, TOF) and any disagreement was resolved by consensus or by discussion with a third reviewer (AR).

The outcomes of interest were clinical and parasitological cure, global response [defined as initial parasitological clearance in combination with clinical improvement, or clinical cure alone for patients for whom a test of cure (TOC) could not be performed], early mortality (reported death during or until 30 days after treatment), treatment interruption due to intolerance and adverse event rate and relapse rate.

The selected articles were read in full to confirm eligibility and to extract data. Data extraction and quality assessment were carried out by one author and checked by a second reviewer (TOF). The following information was recorded: country; year of publication and design of the study, patient characteristics (age, gender, CD4 lymphocyte count, antiretroviral use), rate of primary VL, treatment schedule, VL diagnostic criteria, VL cure criteria (clinical and/or parasitological), loss of following, clinical and parasitological cure rate. The follow-up length and relapse rate were also recorded.

The Newcastle-Ottawa Scale (NOS) [13] was used to assess the quality of nonrandomized studies. Using this 'star system' each included study was judged on three broad perspectives: the selection of the study groups; the comparability of the groups; and the ascertainment of outcome of interest.

Statistical analysis

Comprehensive Meta-Analysis software v.2.2.048 was used to perform one-group meta-analysis of study arms with the same drug to estimate pooled rates of success and adverse events with each drug. These estimates were used, when possible, to indirectly compare treatment options. These unadjusted indirect comparisons were confronted with direct comparisons, when available. Also, the indirect comparisons were adjusted for CD4 lymphocyte count by using meta-regression. Meta-regression was used to explore the relationship between event rate and CD4 count by using mixed effects regression (unrestricted maximum likelihood). We used the Inconsistency (I^2) statistic to evaluate heterogeneity. If significant heterogeneity was found, the results from the random effects model were emphasized and summary measures were analyzed as limited information, looking for differences in studies. Random effects model is a strategy that allows the heterogeneity inter-study would be incorporated through a broad confidence interval, generating a more conservative estimate of the measure of the effect. Clinical cure, global response and death rates analysis were performed according to the intention-to-treat analysis: the analysis was based on the total number of randomly assigned participants, irrespective of how the original study investigators analyzed the data. Publication bias was assessed by Egger's test [14].

Results

Our search identified 342 articles from PubMed. EMBASE, LILACS and WEB OF KNOWLEDGE databases added 10, 2 and 3 papers respectively. After exclusions by title and abstracts, 63 potentially relevant papers were selected for full text evaluation (Figure 1). Two other titles were identified from references of the primary manuscripts. Of these 65 studies, 47 papers were excluded due to incomplete information about treatment and/or outcomes, including two congresses abstracts. One paper [15] was excluded because same patients were described elsewhere [16]. In a study [17], one treatment arm was also excluded by overlapping publication [18]. Thus, we included 17 articles [6,16–31] involving 920 VL episodes among HIV-infected patients. Although in 4 studies the percentage of VL primary and relapse was not informed, most included patients had first VL episodes (76.1%).

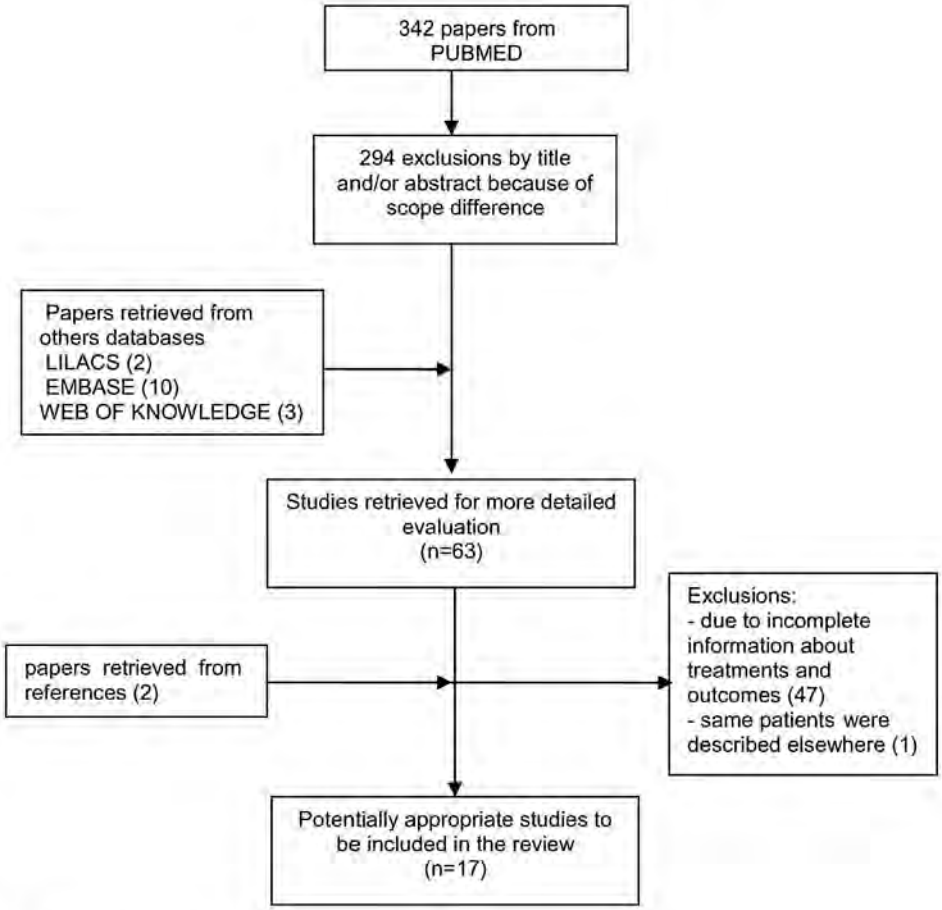

Figure 1. Study selection process.
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From 17 studies, only four were randomized trials [6,22,23,27] involving 279 participants. However, these trials compared different treatment arms. We found only two studies [29,30], nonrandomized, compared the same treatment regimens (amphotericin *versus* low dose antimony) and thus allow direct comparison. The methodological characteristics of studies, namely inclusion and exclusion, VL disease, clinical improvement and TOC criteria are shown in Tables 1 and 2. Three papers were prospective non comparative studies and other nine were historic cohort studies with one or more treatment regimen arms reported.

VL was diagnosed in 10 studies exclusively if patients had a compatible clinical illness and positive Giemsa-stained smears or culture for *Leishmania* in samples taken in most cases from the bone marrow, spleen or liver. Few diagnoses were established by the finding of *Leishmania* spp. in biopsy of an unusual site such skin, tongue and gingival mucosae [29] or after staining and/or culture of the buffy coat [20]. Five authors [6,23,25,26,30] accepted VL diagnosis based on a positive serological result (Western Blot, direct agglutination or rK39 dipstick tests) and in some studies [6,26] [32], diagnosis was based on clinical grounds alone (negative serological tests, strong clinical suspicion and parasitological test contraindicated).

The efficacy of therapy was assessed by clinical and/or microbiologic criteria. The clinical response definition varied among studies. The remission of fever, improvement of hematological values and regression in the size of the spleen were the main signs observed. A complete resolution of all clinical and hematological parameters [16,28,30] or the absence of recurrence in subsequent months after VL treatment [17,32] was required by some authors to establish clinical cure while all the others defined response as the improvement of the signs and symptoms attributed to disease at the end of treatment, even partial.

Table 1. Studies characteristics: design, treatment schedules and therapy arms with available data.

Author, year, country	Treatment arms (number of treated episodes)	Treatment regimens	Study design
Ritmeijer, 2011, Ethiopia	Liposomal amphotericin B (195)	total dose of 30 mg/kg, intravenously, divided into 6 infusions of 5 mg/kg on alternate days	Retrospective, historic cohort, non comparative, multicenter
Sinha, 2011, India	Liposomal amphotericin B (55)	total dose of 20 mg/kg, intravenously, divided into 4 infusions of days 1, 2, 5, and 10 (or patients relapsing after having previously received a full course of liposomal amphotericin B, a total dose of 25 mg/kg was given in 5 doses: days 1, 2, 5, 10, and 15)	Retrospective, historic cohort, non comparative, multicenter
Molina, 2007, Spain	Liposomal amphotericin B (24)	4 mg/kg/day intravenously for 5 consecutive days and once per week thereafter for 5 more weeks (total, 10 doses 40 mg/kg).	Prospective, non-comparative, single center
Ritmeijer, 2006, Ethiopia	Miltefosine (63)	100 mg per day for 28 days, orally	Prospective, randomized, open label, multicenter
	Sodium stibogluconate (44)	20 mg/kg per day by intramuscular for 30 days.	
Laguna, 2003, Spain	Amphotericin B lipid complex (17)	total dose of 15 mg/kg , intravenously, 3 mg/kg/day for 5 days	Prospective, randomized, open label
	Amphotericin B lipid complex (20)	total dose of 30 mg/kg , intravenously, 3 mg/kg/day for 10 days	
	Meglumine antimoniate (19)	20 mg Sbv/kg/day, intramuscularly, for 28 days	
Ritmeijer 2001, Ethiopia	Generic sodium stibogluconate or Pentostam (27)	both drugs were given at 20 mg/kg intramuscularly for 30 days	Prospective, randomized, open label
Pintado, 2001, Spain§ #.	Meglumine antimoniate (51)	20 mg Sbv/kg/day (with a maximum daily dose of 850 mg), intramuscularly for 3–4 weeks	Retrospective, historic cohort, single center
Laguna, 1999, Spain	Meglumine antimoniate (44)	20 mg Sbv/kg/day (without an upper dose limit), intramuscularly, for 28 days.	Prospective, randomized, open label, multicenter
	Amphotericin B deoxycholate (45)	0.7 mg/kg/day for 28 days, intravenously	
Behre, 1999, Ethiopia	Meglumine antimoniate (23)	20 mg Sbv/kg/day intramuscularly for 28 days	Prospective, cohort, non-comparative
Delgado, 1999, Spain#	Meglumine antimoniate (25)	20 mg Sbv/kg/day given in 2 separate intramuscular injections (without an upper dose limit)	Retrospective, historic cohort, comparative
López-Veléz, 1998, Spain¶ #	Meglumine antimoniate (51)	20 mg/Sbv/kg/day given intravenously or intramuscularly for 28 days &	Retrospective, historic cohort, comparative
Laguna, 1997, Spain§	Meglumine antimoniate (29)	≈20 mg Sbv/kg/day for at least 28 days, intramuscularly	Retrospective, historic cohort, comparative
	Liposomal Amphotericin B (4)	NA	
Delgado, 1997, Spain#	Meglumine antimoniate (21)	three-week course 20 mg Sbv/kg/day (with a maximum daily dose of 850 mg), intramuscularly	Retrospective, historic cohort, comparative
	Amphotericin B deoxycholate (20)	0,5 mg/Kg/day (total dose 1–1.5 g)	
Russo, 1996, Spain, Portugal and Italy	Liposomal amphotericin B (10)	Total dose of 40 mg/kg, intravenously, 4 mg/kg on days 1, 2, 3, 4, 5, 10, 17, 24, 31, 38.	Prospective, non-comparative, multicenter
Ribera, 1996, Spain	Meglumine antimoniate (52)	three week course of daily dose 850 mg, intravenously or intramuscularly	Retrospective, historic cohort non comparative, single center
Rosenthal, 1995, France¶	Meglumine antimoniate (27)	20 mg Sbv/kg/day for at least 21 days, intravenously or intramuscularly	Retrospective, historic cohort comparative, multicenter
	Amphotericin B deoxycholate (14)	≈20 mg/kg total dose, intravenously	
Montalban, 1990, Spain	Meglumine antimoniate (40)	three-week course of daily dose 850 mg, intravenously or intramuscularly	Retrospective, historic cohort non comparative, multicenter

NA: information not available **Sbv**: pentavalent antimony.
#in some cases therapy was combined with oral allopurinol (300–1,200 mg/day), or interferon-γ (100 μg/m2 subcutaneously) **x̃**: median **μ**: mean **SD**: standard deviation.
¶one or more treatment arms excluded due to absence separated data.
§one treatment arm (meglumine antimoniate low dose) excluded: patients data published elsewhere and another (amphotericin B) due to absence separated data.
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A VL episode was considered microbiologically cured when the organ used at inclusion and obtained again usually between days 25 and 45 after initiation of therapy yielded no demonstrable amastigotes by direct visualization or culture. A parasitological control study was planned for all patients in three prospective trials [21,22,27]. Only two studies did not report parasitological test of cure [18,29]. In several centers [6,23,25,26] parasitological test of cure have been routinely performed after treatment, except in

Table 2. Definition criteria used by the studies.

Author, year	Exclusion criteria	VL diagnosis criteria	Clinical cure criteria	Parasitological test of cure
Ritmeijer, 2011	Treatment combination with another antileishmanial drug, patients switched to another treatment due to intolerance	Parasitologically or serologically (rK39 and/or DAT) confirmed, and in few cases clinically defined (negative serological test but strong clinical suspicion of VL and parasitological exam contraindicated).	Fever resolution, spleen regression, hemoglobin increase, and weight gain	Planned for all patients on day 28, if could not be done because of absence of palpable spleen or lymph nodes
Sinha, 2011	NA	Parasitologically or serologically (rK39 and/or DAT) confirmed after exclusion of malaria and bacterial infection.	Clinical improvement	Planned for all patients at 1 month after treatment initiation. It was not performed in clinically cured patients, patients presenting late or material not available
Molina, 2007	Age <18 years, patients without HAART and/or secondary prophylaxis after VL treatment, non-liposomal amphotericin treatment	Parasitologically confirmed	Resolution of fever and improvement of the hematological parameters	Assessment of cure was decided by the attending physician and was performed 1 month after completing treatment
Ritmeijer, 2006	Females, males aged <15 years, severe comorbidity (patients considered to be likely to die during the month's treatment)	Parasitologically or serologically (DAT) confirmed	Clearance of fever, in combination with spleen regression, increased hemoglobin, or weight gain	Splenic or lymph node aspirate performed at day 27–30. In patients without palpable spleen or lymph nodes, cure was established only clinically
Laguna, 2003	Pregnant women, women at risk for pregnancy or were lactating, patients with pancreatitis, prothrombin activity <40%, aminotransferase levels 10× the upper normal limit, myocardiopathy, heart failure, a Qt corrected interval >500 ms, creatinine levels >twice the upper normal limit, allergy to either ABLC or meglumine antimoniate, concomitant treatment with dideoxycytidine or dideoxyinosine and a life expectancy of <6 months.	Parasitologically confirmed	NA	Tissue biopsy sample, taken from the organ used at inclusion (bone marrow, spleen or liver) between 1 and 7 weeks after the completion of therapy
Ritmeijer, 2001	Patients previously treated for VL	Parasitologically or serologically (rK39 and/or DAT) confirmed and in few cases clinically defined (negative serological test but strong clinical suspicion of VL and parasitological exam contraindicated)	Resolution of fever, spleen regression, and weight gain	Performed in patients with splenomegaly at day 25–30
Pintado, 2001	NA	Parasitologically confirmed and in few cases clinically defined (suggestive clinical features, significant serologic titers, and response to specific treatment)	Resolution of fever, improvement of the pancytopenia and hepatosplenomegaly, and absence of symptoms 1 month after the end of treatment	Performed in some patients within first month after the completion of treatment
Laguna, 1999	Age <18 years, pregnant women, history of hepatic encephalopathy, ascites, pancreatitis, myocardiopathy or heart failure, prothrombin time of 20 s or greater, aminotransferase levels 10 times the upper normal limit or higher, or a serum creatinine level above 2 mg/dl.	Parasitologically confirmed	Resolution of fever, improvement of hematological values and regression in the size of the spleen (when it was palpable)	Planned for all patients 4 weeks and 12 months after the completion of therapy
Behre, 1999	Other obvious concurrent infectious diseases	Parasitologically confirmed	Improvement of general condition, reduction in spleen size	Performed in all patients after completion of anti Leishmania chemotherapy
Delgado, 1999	Non antimonial treatment	Parasitologically confirmed	Resolution of all symptoms and signs attributable to VL	Performed in some patients following completion of therapy
López-Veléz, 1998	Treatment response not available	Parasitologically confirmed	Resolution of fever, improvement of hematological and hepatosplenomegaly, and absence of symptoms 3 weeks after the end of treatment	Performed (Without any selective criteria) on some patients one month after completing the treatment
Laguna, 1997	NA	Parasitologically confirmed	Remission of clinical symptoms potentially due to VL and the absence of any VL relapse for at least six months after treatment.	Criteria used not available

Table 2. Cont.

Author, year	Exclusion criteria	VL diagnosis criteria	Clinical cure criteria	Parasitological test of cure
Delgado, 1997	NA	Parasitologically confirmed	Remission of clinical symptoms potentially due to VL	NA
Russo, 1996	NA	Parasitologically confirmed	Improvements in weight, albumin, pancytopenia, and erythrocyte sedimentation rate	Performed on day 45 using criteria not reported
Ribera, 1996	Age <14 years	Parasitologically confirmed	Normalization of all the clinical and hematological parameters	Performed in all patients who showed any persistent clinical or hematological alterations after treatment and in some cases with recovery clinical criteria
Rosenthal, 1995	Incomplete treatment course	Parasitologically confirmed (one case was serologically confirmed by Western Blot)	Disappearance of all clinical signs potentially due to VL	Not reported in some patients because of absence of clinical or microbiological data
Montalban, 1990	NA	Parasitologically confirmed	NA	NA

NA: information not available **VL**: visceral leishmaniasis **Parasitologically confirmed:** identification of *Leishmania* amastigotes by direct examination or by isolation of promastigotes in culture of tissue samples **NA**: information not available.
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those patients without splenomegaly and lymphadenopathy or contraindication to the procedure. In another study [16], the procedure was carried on patients who showed any persistent clinical or hematological alterations after treatment and finally there were those where TOC was performed in some patients after completion of therapy without any disclaimed selective criteria. The percentage of VL episodes tested for parasitological cure ranged from 7.4 to 100% among studies. Overall, 62.3% (456) of the 732 treated episodes with available information about TOC were evaluated with a parasitological test at the end of treatment. Fourteen studies reported number of patients lost to follow up: in 13 (93%) of them it was less than 10%. The follow up length (mean or median reported by fifteen studies) varied between 5 and 14 months (Table 3).

Table 3 shows also the baseline characteristics of included patients. The mean or median age varied between 28 and 36 years among the studies, most patients were male (87.4% of the 748 patients with gender information available) and 49.6% of the patients (269/542) had AIDS criteria before VL diagnosis. Antiretroviral use was reported in 10 studies and in four of them, no patient was on HIV treatment at VL diagnosis. The median or mean baseline CD4 cell counts range from 25 to 204 cells/ml.

Five treatment regimens were reported in 17 studies included in this review. There were 13 studies with 457 VL episodes evaluating antimony compounds: five studies assessing "low antimonial dose" and eight evaluating "high antimonial dose" schedules. Therefore, we assume that a low pentavalent antimony dose was administered when the length of treatment was shorter than 28 days and/or less than 20 mg Sbv/kg/day was administered. High antimonial dose was defined as treatment with ≥20 mg Sbv/Kg/day for at least 28 days. In some studies, the antimonial treatment was combined with allopurinol, or recombinant human interferon-gamma in few patients [20,28,29,31]. Three studies evaluated the response to amphotericin B deoxycholate and 6 studies described the response to one of the lipid formulations of amphotericin B (L-Lip-AmB), which includes liposomal and lipid complex amphotericin, most of them (5 of 6) by using total doses above 25 mg per kg. Only one study evaluated the response to treatment with miltefosine [23].

The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomized Studies is show in Table 4 and the scores ranged from 5–7. As shown in Table 2, there was adequate selection of patients in included studies, as almost all were parasitological confirmed cases who were largely representative of source populations. In ten studies, however, patients were reviewed retrospectively for inclusion, with some risk of bias in the case selection. Seven studies were non comparative and reported only one arm treatment outcome.

The summarized measures for initial clinical improvement, global cure and death, according to the intention-to-treat analysis, are shown in Table 5. Relapse was assessed including treated patients who were considered cured. To assess parasitological cure rate only patients who underwent test of cure were included.

Clinical improvement rate using amphotericin in lipid formulation (L-Lip-AmB) was superior compared to both antimony therapy groups (Figure 2).The unique study herein included using deoxycholate amphotericin B also exhibited a clinical response rate (85%, 95%CI 41.7 97.8%) similar to L-Lip-AmB group (91.6%, 95%CI 74.7 97.6%). Therefore, it was not possible to attest the presence of a difference in performance among several amphotericin B formulations. The global and parasitological cure rates varied widely within the same treatment arm, which hampered any indirect comparison between them (Table 5). This fact probably reflects different criteria used by the studies to perform test of cure. However, it is worth mentioning that the difference by indirect comparison from 76% to 56% (wide confidence intervals) in global cure rate between deoxycholate amphotericin B and Sbv treatment groups, respectively, although heterogeneous, was confirmed by direct comparison of the studies [29,30] which actually compared these two treatment arms (OR 6,08 for amphotericin superiority 95%CI 1,99 18,5; I^2 0%).

Regarding tolerance, the difference in adverse event rate between high dose of Sb^v (23.3; 95%CI 17.4 30.4) and lipid formulation of amphotericin B (9.5; 95%CI 3.5 23.3) seems to be relevant, despite the overlap between the confidence intervals observed (Table 5). In agreement with this, the rate of early discontinuation of therapy due to toxicity also seems to be higher with Sb^v than with lipid formulation of amphotericin B. All these

Table 3. Main patient characteristics.

Author, year, country	Treatment arms (number of episodes treated)	Age (min-max) years ± SD	Male/female	CD4 cell count (min-max) cells/mm³	Antiretroviral therapy use (% of patients)	Previous AIDS diagnosis (% of patients)	% first VL episode	Follow up (interquartile range or standard deviation) in months	Lost to follow up (%)	Treated patients with parasitological test of cure (%)
Rijmeijer, 2011, Ethiopia	Liposomal amphotericin B (195)	μ = 30 (15–56)	179/16	X̄ = 155±123	42/87 (48.3)	NA	59.5	NA	3/195 (1.5)	129/195 (66.1)
Sinha, 2011, India	Liposomal amphotericin B (55)	μ = 35 (30–40)	46/9	μ = 66 (38–112)	0/55 (0)	NA	50.9	x̄ = 14 (3.4–20.3)	0/55 (0)	43/55 (78.2)
Molina, 2007, Spain	Liposomal amphotericin B (24)	μ = 36 (26–53)	14/1	x̄ = 100 (4–300)	13/15 (86.7)	7/12 (58.3)	57	x̄ = 14 (5–44)	2/24 (8.3)	12/24 (50)
Rijmeijer, 2006, Ethiopia	Miltefosine (63)	x̄ = 33.4±9.5	NA	NA	NA	NA	NA	6	NA	NA
	Sodium stibogluconate (44)									
Laguna, 2003, Spain	Amphotericin B lipid complex (17)	x̄ = 34.1±6	15/3	x̄ = 41 (5–366)	5/18 (27.8)	10/18 (55.6)	100	5	5/56 (8.9)	43/56 (76.7)
	Amphotericin B lipid complex (20)	x̄ = 33.7±5.8	17/3	x̄ = 56 (0–731)	13/20 (65)	13/20 (65)				
	Meglumine antimoniate (19)	x̄ = 34±5.2	16/3	x̄ = 26 (1–600)	7/19 (36.8)	16/19 (84.2)				
Rijmeijer, 2001, Ethiopia	Generic sodium stibogluconate or Pentostan (27)	x̄ = 29.9	25/2	NA	NA	NA	100	6	1/27 (3.7)	2/27 (7.4)
Pintado, 2001, Spain	Meglumine antimoniate (51)	x̄ = 33.2±8.2	64/16	32/80 <200	19/80 (23.8)	43/80 (53.8)	100	μ 13.8 (3–44)	NA	30/51 (58.8)
Laguna, 1999, Spain	Meglumine antimoniate (44)	μ = 31 (19–64)	40/4	μ = 29 (1–203)	NA	28/44 (63.6)	100	x̄ 10.8	6/89 (6.7)	58/89 (65.2)
	Amphotericin B deoxycholate (45)	μ = 32 (22–57)	36/9	μ = 18 (0–231)		28/45 (62.2)				
Behre, 1999, Ethiopia	Meglumine antimoniate (23)	x̄ = 28.6 (20–47)	22/1	NA	0/23 (0)	NA	NA	NA	0/23 (0)	23/23 (100)
Delgado, 1999, Spain	Meglumine antimoniate (25)	x̄ = 30.2 (19–39)	20/4	x̄ = 66.2 (2–307)	0/25 (0)	17/24 (70.8)	96	13.5 (0.25–26)	1/25 (4)	9/25 (36)
Lopez-Velez, 1998, Spain	Meglumine antimoniate (51)	x̄ = 31±7.5	46/8	33/54 <100	NA	25/54 (46.3)	NA	6	0/51 (0)	14/51 (27.4)
Laguna, 1997, Spain	Meglumine antimoniate (29)	NA	42/1	34/43 <100	0/33	29/43 (67.4)	70	μ = 10.4 (2–25)	3/33(9.1)	23/33 (69.7)
	Liposomal Amphotericin B (4)									
Delgado, 1997, Spain	Meglumine antimoniate (21)	x̄ = 34.3±5.1	30/1	x̄ = 37.9 (0–139)	NA	18/31 (58)	75.6	μ = 10.9±8.5	NA	0
	Amphotericin B deoxycholate (20)									
Russo, 1996, Spain, Portugal and Italy	Liposomal amphotericin B (10)	x̄ = 31.3 (25–43)	9/1	NA	NA	5/10 (50)	40	μ = 12	0/10 (0)	8/10 (80)
Ribera, 1996, Spain	Meglumine antimoniate (52)	x̄ = 28 (20–64)	NA	x̄ = 26.7 (0–268)	37/52 (71.1)	18/52 (34.6)	71.7	x̄ = 7.5	0/52 (0)	26/52 (50)
Rosenthal, 1995, France	Meglumine antimoniate (27)	x̄ = 34 (22–68)	42/8	μ = 23 (0–200)	NA	21/50 (42)	NA	NA	5/41 (12.2)	36/41 (87.8)
	Amphotericin B deoxycholate (14)									
Montalban, 1990, Spain	Meglumine antimoniate (40)	x̄ = 29.4 (20–53)	36/4	x̄ = 204.3±27.5	NA	19/40 (47.5)	NA	μ = 12.8 (0.5–71)	0/40 (0)	0

VL: Visceral leishmaniasis **NA:** information not available **x̄:** median **μ:** mean **SD:** standard deviation.

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outcomes were adjusted for CD4 lymphocyte count, which had no influence on treatment effect as evaluated by meta-regression.

The most outstanding difference in outcome between the treatment regimens was observed in early mortality rate: about 3 times higher in high-dose antimony (18.4%, 95%CI 13.3 25%) in comparison to L-Lip-AmB (6.1%, 95%CI 3.9 9.4%) treated patients, without overlap of confidence intervals (Table 5 and Figure 3). Meta-regression revealed no influence of CD4 lymphocyte count in death rate related to different treatments (Figure 4).

A variety of adverse effects were depicted, such as vomiting, diarrhea, anemia, eletrolic disturbs, pancreatic, cardiac, hepatic and renal dysfunction (Table 6). The events reported were not sufficiently similar to allow a meta-analysis of adverse effects. In two studies [22] [27] adverse reactions were scored according to the World Health Organization (WHO) scale for toxicity. Six studies did not report on the occurrence of reactions to VL therapy and one study [17] reported the occurrence of adverse events without discriminating the type of treatment received.

Ten out seventeen studies reported the VL relapse rate without secondary prophylaxis and it ranged from 26 to 50%. VL relapse was diagnosed if parasites were observed in tissue samples after initial clinical cure. It was not possible to attest the presence of any difference in relapse among different treatments.

Discussion

Available evidence suggests superiority of amphotericin B in the treatment of HIV-infected patients with visceral leishmaniasis (VL-HIV). The main conclusion of this review is the higher mortality rate among VL-HIV patients treated with Sb^V than among patients treated with amphotericin B. It could be due to the low efficacy or the toxic effects of antimony; however the risk of death seems to be related to the increase in Sb^V dose, suggesting that toxicity is the most important factor. Our data confirm that antimony compounds are poorly tolerated in the presence of HIV infection, as clearly demonstrated by the study evaluating mortality among coinfected and HIV-uninfected, both treated with Sb^V [23]. The higher mortality related to Sb^V than that observed with miltefosine in HIV-infected patients strongly suggests that this effect was caused by the antimonial treatment itself. Pentavalent antimonial drugs have been used for the treatment of VL since the 1940s [33]. Sodium stibogluconate (brand name Pentostam [GSK]); also generic versions from many manufacturers) and meglumine antimoniate (brand name Glucantime [Aventis]) remain the most widely used antileishmanial agents [33]. The mechanism of action of pentavalent antimonial drugs is uncertain; *in vivo* conversion to trivalent antimony compounds may be involved in both antileishmanial activity and drug toxicity [34]. Other studies suggest that antileishmanial activity may occur via inhibition of parasite ADP phosphorylation, DNA I topoisomerase, and/or trypanothione reductase [35]. The compounds are well known for their toxicities such as severe vomiting, arrhythmia and pancreatitis, besides emerging drug resistant. Doses below those currently used (such as three weeks course with a maximum daily dose of 850 mg of Sb^V in adults) have been used in the past [18,30,31] but were abandoned by lower efficacy compared to 20 mg/kg/day of Sb^V for a minimum of 28 days, which was also suggested by our data. In this review, it was also observed that, as is already accepted [36,37], toxicity is directly related to the increase in the dose of Sb^V, verified by an increase in the occurrence of severe events and mortality rate. Although sensitivity to specific drugs varies by region, it is unlikely that these regional differences and strains of *Leishmania* have contributed to the discrepancy observed in clinical response since all except one study were performed in Europe and Ethiopia, where resistance is rare. Only one study performed in India was included and, in this case, the treatment was carried out with liposomal amphotericin B and it reached a clinical response rate of 93% [25]. Meanwhile it is not possible to certify that other variables related to the characteristics of patients in different countries, such as comorbidities or degree of immunosuppression, or to clinical spectrum of the disease, have influenced the efficacy and mortality results.

All but one study in the L-Lip-AmB group used liposomal formulation as treatment. Liposomal amphotericin B (AmBisome, Gilead) consists of amphotericin B packaged with cholesterol and other phospholipids within a small unilamellar liposome. The liposomal drug formulation has improved stability in blood, macrophages, and tissues, permitting more effective tissue penetration with sustained tissue drug levels, especially in the liver and spleen. This formulation has increased affinity for ergosterol and its precursors. In addition, the presence of cholesterol in the formulation minimizes interaction with mammalian cell membranes, thereby reducing toxicity [38]. Because cost is the limiting factor for use of liposomal amphotericin B, many different regimens have been evaluated in an attempt to find the lowest total dose with acceptable efficacy. Due to the small number of studies in this review, it was not possible to compare schemes with different doses or preparations of amphotericin B. Our data suggest, however, that liposomal and lipid complex preparations are better tolerated than conventional amphotericin B or pentavalent antimony, and this probably contributes to their highest rate of clinical efficacy. Current research is now shifting away from developing optimized regimens of existing drugs toward demonstrating their implementation is feasible in the field [39]. There is a clear trend among experts that antileishmanial therapy in endemic regions should move toward combination drug regimens based on the following rationale: (1) protect the limited armamentarium of antileishmanial agents from development of acquired resistance and (2) establish shorter treatment courses with high efficacy to improve compliance and decrease treatment costs [36]. Drugs for Neglected Diseases initiative (DNDi), a collaborative, patients' needs-driven, non-profit drug research and development organization is currently funding several studies in Eastern Africa, including two randomized, open-label clinical trials assessing the safety and efficacy of combination regimens: SSG plus single dose AmBisome, miltefosine plus single dose AmBisome (ClinicalTrials.gov NCT01067443) and SSG plus paramomycin sulphate (ClinicalTrials.gov NCT00255567). As far as we know, there is no registered trial evaluating combination therapy for *Leishmania*-HIV coinfected patients.

Limitations

The main limitation of this review is the paucity of quality evidence. On the other hand, four literature databases were searched, making it a comprehensive review. Clinical decisions must be made. To aid in this task we presented indirect comparisons, including non-randomized studies, in the same way others have done [40], as a tool to synthesize the available information. By adjusting indirect comparison for CD4 lymphocyte count, we have evaluated an important confounder factor for mortality rate. To our knowledge, no systematic review has investigated the comparative efficacy of the several treatment options for VL-HIV patients.

When there is no or insufficient direct evidence from randomized trials, the adjusted indirect comparison may provide useful or supplementary information on the relative efficacy of

Table 4. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies.

Non RTC studies	Selection			Comparability			Assessment of Outcome			Total Quality score
Author, year	Representativeness of treated arm	Selection of the comparative treatment arm (s)	Ascertainment of the treatment regimen	Demonstration that outcome of interest was not present at star of study	Comparability between patients in different treatment arms - main factor: CD4 lymphocytic count	Comparability between patients in different treatment arms - secondary factor: co-morbidities	Assessment of outcome with independency	Adequacy of Follow up length (to assess outcome)	Lost to follow up acceptable (less than 10% and reported)	
Ritmeijer, 2011	*	*	*	*		*	*	*	6	
Sinha, 2011	*	*	*			*	*	*	6	
Molina, 2007	*	*	*			*	*	*	6	
Pintado, 2001	*	*	*			*	*		5	
Laguna, 1999	*	*	*			*	*	*	6	
Behrj, 1999	*	*	*			*	*	*	6	
Delgado, 1999	*	*	*			*	*	*	6	
López Vélez, 1998	*	*	*			*	*	*	7	
Laguna, 1997	*	*	*			*		*	6	
Delgado, 1997	*	*	*			*	*		6	
Russo, 1996	*	*	*			*	*	*	6	
Ribera, 1996	*	*	*			*	*	*	6	
Rosenthal, 1995	*	*	*			*	*		6	
Montalban, 1990	*	*	*			*	*	*	6	

RTC: randomized controlled trial.
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Table 5. Summarized measurements (95% confidence interval) for main outcomes.

Treatment group	Clinical cure rate %	Parasitological cure rate %	Global cure rate %	Early death rate %	Relapse rate %	Serious adverse event rate %	Treatment interruption due to intolerance %
Antimony low dose	58.4 (33.0–80.0)	98.1 (76.4–99.9)	52.2 (30.2–73.3)	7.2 (3.6–14.1)	40.6 (17.2–69.3)	4.8 (0.7–27.1)	10.8 (3.8–27.4)
	$I^2 = 73.8$		$I^2 = 0$	$I^2 = 0$	$I^2 = 0$		$I^2 = 36.9$
	n = 75 (3 studies)	n = 26 (1 study)	n = 99 (3 studies)	n = 113 (3 studies)	n = 50 (2 studies)	n = 21 (1 study)	n = 124 (3 studies)
Antimony high dose	58.7 (35.9–78.4)	72.6 (55.4–85)	56.1 (40.7–70.4)	18.4 (13.3–25)	25.6 (12.9–44.6)	23.3 (17.4–30.4)	21 (9.2–41.2)
	$I^2 = 87.8$	$I^2 = 28.4$	$I^2 = 76.9$	$I^2 = 44.8$	$I^2 = 55.6$	$I^2 = 66.3$	$I^2 = 80.4$
	n = 126 (4 studies)	n = 121 (2 studies)	n = 211 (2 studies)	n = 192 (6 studies)	n = 58 (3 studies)	n = 456 (5 studies)	n = 131 (4 studies)
Amphotericin B deoxycholate	85.0 (41.7–97.8)	62.2 (47.4–75.1)	76.8 (54–90.3)	11.1 (4.7–24.1)	33.1 (7.6–53.9)	16.6 (9.5–27.2)	10.6 (3.0–31.5)
			$I^2 = 58.5$			$I^2 = 0$	$I^2 = 0$
	n = 20 (1 study)	n = 45 (1 study)	n = 79 (3 studies)	n = 45 (1 study)	n = 24 (1 study)	n = 290 (2 studies)	n = 65 (2 studies)
L-Lip-amB doses <2.5 mg/Kg	-	37.5 (17.9–62.3)	35.3 (16.8–59.6)	2.8 (0.2–32.2)	50 (12.3–87.7)	2.8 (0.2–32.2)	5.6 (0.8–30.7)
		n = 16 (1 study)	n = 17 (1 study)	n = 17 (1 study)	n = 4 (1 study)	n = 17 (1 study)	n = 18 (1 study)
L-Lip-amB doses >2.5 mg/Kg	91.6 (74.7–97.6)	72.3 (51.0–86.7)	72.7 (56–84.8)	6.1 (3.9–9.4)	39.4 (18.9–64.5)	9.5 (3.5–23.3)	4.2 (1.3–14.9)
	$I^2 = 0$	$I^2 = 74.6$	$I^2 = 80.7$	$I^2 = 0$	$I^2 = 74.2$	$I^2 = 0$	$I^2 = 0$
	n = 89 (3 studies)	n = 213 (6 studies)	n = 318 (6 studies)	n = 308 (6 studies)	n = 68 (4 studies)	n = 51 (3 studies)	n = 106 (4 studies)
Miltefosine	-	-	77.8 (65.9–86.4)	5.6 (0.2–10.4)	28.6 (18.3–41.7)	24.6 (17.9–32.9)	10.8 (10.1–11.3)
			n = 63 (1 study)	n = 49 (1 study)	n = 56 (1 study)	n = 126 (1 study)	n = 63 (1 study)

I^2 values of <25%, 25 to 50% and >50% indicate mild, moderate and substantial heterogeneity, respectively

n: number of patients available

L-Lip-amB: Lipid formulations of amphotericin B.

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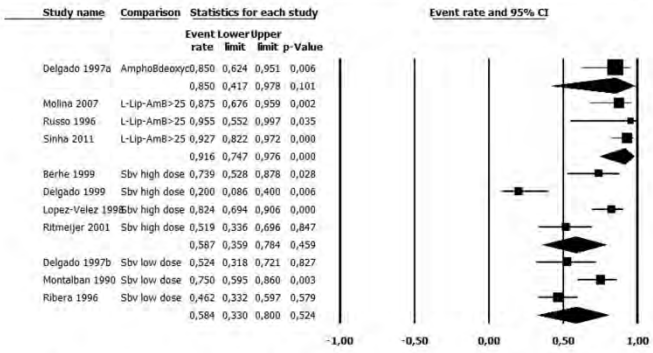

Figure 2. Clinical improvement rate. Egger's test for publication bias (all studies): p = 0.18 Statistical heterogeneity: I^2 (L-Lip-AmB>25) = 0; I^2 (Sbv high dose) = 87.8; I^2 (Sbv low dose) = 73.
doi:10.1371/journal.pntd.0002195.g002

competing interventions. The validity of the adjusted indirect comparisons depends on the internal validity and similarity of the included trials [41]. Ideally, direct and indirect estimates should be combined in mixed treatment comparisons only after adequate assessment of the consistency of the evidence [42]. In this case, evidence of consistency is the correlation observed between indirect comparison performed (comparison among patients treated with different schemes in different studies) and the only direct comparison that could be made (two studies comparing the same two treatment arms). Other important qualitative features include the degree of similarity of populations, interventions, outcomes, study objectives and study designs that incorporate both clinical and biological plausibility.

Many studies used selective criteria excluding patients with more severe clinical conditions or with high risk of toxicity, such as those with renal, pancreatic or heart dysfunction. This methodological choice could have influenced toward a lower rate of adverse events and a higher percentage of therapeutic success. However, the more stringent studies were also comparative and randomized studies [22,23,27], so, such selection affected equally all treatment arms. Similarly, studies with highly demanding criteria of cure, as those requiring complete resolution of symptoms [16,30] may have had the therapeutic success rates underestimated.

In all studies, the outcome ascertainment was not blinded. Indeed, either the participants or the researchers who collected

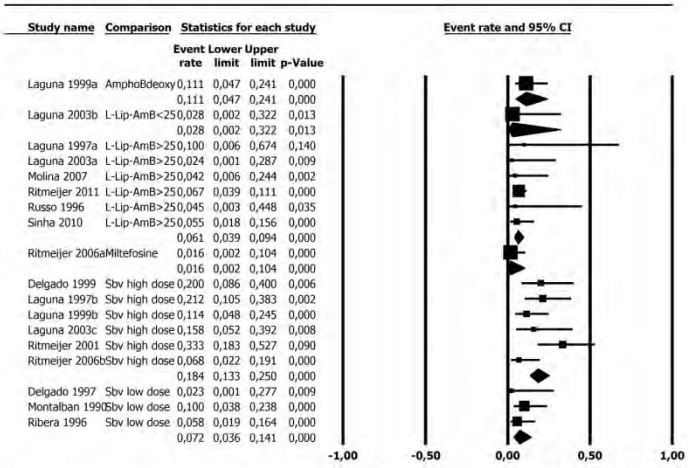

Figure 3. Death rate. Egger's test for publication bias (all studies): p = 0.20 Statistical heterogeneity: I^2 (L-Lip-AmB>25) = 0; I^2 (Sbv high dose) = 44.8; I^2 (Sbv low dose) = 0.
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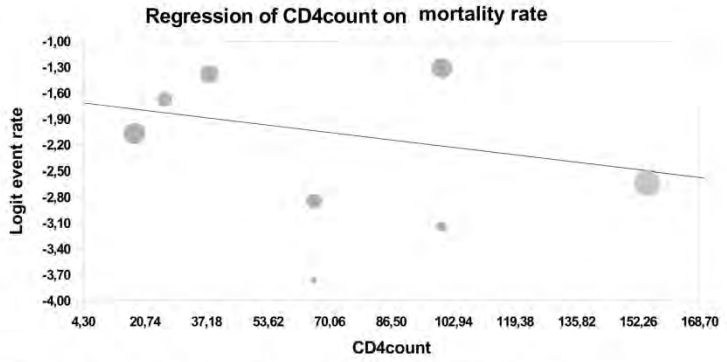

Figure 4. Meta-regression between death rate and CD4 lymphocytes count. p = 0.18.
doi:10.1371/journal.pntd.0002195.g004

disclosure information may have been aware of participants' disease status at the time of data extraction. However, most studies have clearly defined criteria for establishing cure, and in many of them parasite clearance was required. The time to cure assessment and the follow-up time was relatively uniform and adequate in all studies. So we performed the summary measures of effectiveness for each treatment regimen to perform an indirect efficacy comparison. To carry out a clinically sound analysis, we used a conservative approach and imputed outcomes for the missing and discontinued participants assuming that they did not respond to treatment. Therefore, no response includes the intrinsic lack of efficacy and toxicity limiting the completeness of the treatment. In fact, in this review there was an inverse association between adverse events rate and clinical response, as expected. Although parasitological cure rate could theoretically provide reliable information about treatment efficacy, in most studies post treatment TOC was performed only in patients with uncertain clinical response, which represents a selection bias that could underestimate response rates.

Table 6. Adverse events reported.

Author, year	Adverse events
Ritmeijer, 2011	NA
Sinha, 2011	NA
Molina, 2007	Nonsystematic description of the adverse effects observed. Only impairment of renal function was reported.
Ritmeijer, 2006	It were reported: bleeding, diarrhea, vomiting, pneumonia, death, default#
Laguna, 2003	Adverse reactions were scored according to the World Health Organization (WHO) scale for toxicity. Adverse events were considered to be toxicity of grade 2 or greater
Ritmeijer, 2001	It were reported: bleeding, diarrhea, vomiting, pneumonia, death
Pintado, 2001	NA
Laguna, 1999	Adverse reactions were scored according to the World Health Organization (WHO) scale for toxicity. Adverse events were considered to be toxicity of grade 2 or greater
Behre, 1999	NA
Delgado, 1999	It were reported: hyperamylasemia, acute pancreatitis, serum creatinine >2 mg/dl, leukocyte count <1,500 cells/ml, T wave inversion, vomiting
Lopéz-Veléz, 1998	It were reported the following serious adverse effects: anemia (defined as a 25% reduction in the hematocrit), renal toxicity (a three-fold increase in the normal level of serum creatinine), hepatic toxicity (a ten-fold increase in the base values of the transaminases), and hyperamylasemia (a two-fold increase in normal serum amylase values)
Laguna, 1997	NA
Delgado, 1997	Nonsystematic description of the adverse effects observed
Russo, 1996	Nonsystematic description of the adverse effects observed
Ribera, 1996	NA
Rosenthal, 1995	NA
Montalban, 1990	NA

NA: information not available.
#defined as starting but failing to complete treatment because of reasons other than death or decision by the clinician.
doi:10.1371/journal.pntd.0002195.t006

In conclusion, these indirect comparisons suggest higher clinical response rate with amphotericin B than with antimony treatment, which appears to be related to less toxicity than with higher effectiveness of lipid formulations of amphotericin. Antimonial therapy carries a higher rate of drug discontinuation and a significantly higher mortality indirectly compared to treatment with amphotericin B. A relatively large body of non-comparative cohort studies supports, at this time, the use of amphotericin B as the first choice for VL treatment in HIV-infected patients. The optimal dose of amphotericin and the difference in efficacy between its various formulations remain to be established.

Supporting Information

Checklist S1 PRISMA Checklist.
(DOC)

Author Contributions

Revised the manuscript critically: AR. Conceived and designed the experiments: GFC MRdS AR. Performed the experiments: GFC TOF MRdS. Analyzed the data: GFC MRdS. Contributed reagents/materials/analysis tools: GFC MRdS. Wrote the paper: GFC MRdS.

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PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2, 3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Figure 1
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5-7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6

ARTIGO 4

Submetido

"Comparison of parasitological, serological and molecular tests for visceral leishmaniasis in HIV-infected patients: a cross-sectional delayed type study"

Comparison of parasitological, serological and molecular tests for visceral leishmaniasis in HIV-infected patients: a cross-sectional delayed-type study

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Abstract

The aim of this study was to evaluate the accuracy of invasive and noninvasive tests for diagnosis of visceral leishmaniasis (VL) in a large series of HIV-infected patients. In this delayed-type cross-sectional study, 113 HIV-infected symptomatic patients were evaluated by an adjudication committee after clinical follow-up to establish the presence or absence of VL as the target condition (reference test). The index tests were: recombinant K39 antigen-based immunochromatographic test (rK39), indirect fluorescent antibody test (IFAT), prototype kit

of direct agglutination test (DAT-LPC) and real-time polymerase chain reaction (qPCR) in peripheral blood. Comparing to parasitological test and adjudication committee diagnosis, or through latent class model analyses, IFAT and rK39 dipstick test presented the lowest sensitivity. DAT-LPC exhibited good overall performance and there was no statistical difference between DAT-LPC and qPCR diagnosis accuracy. Real time PCR emerges as a less invasive alternative than parasitological examination for confirmation of cases not identified by DAT.

Keywords: sensitivity and specificity, accuracy, visceral leishmaniasis, diagnosis**,** HIV infection

INTRODUCTION

Concurrent visceral leishmaniasis (VL) and HIV infection have been reported in most areas of the world where the geographical distributions of the two infections overlaps. The disease is characterized by significantly lower cure rates, higher drug toxicity, relapse and mortality rates than those for VL in non-HIV-infected individuals.[1] The clinical diagnosis has many limitations because features of VL can be easily mistaken for other febrile illnesses such as tuberculosis, histoplasmose, enteric fever and lymphoma.[2, 3] Cytopenia is frequent during the course of HIV infection and may result from several mechanisms.[4] In addition, it is important to be alert for possible situations of co-infection where manifestations of VL are atypically present.[5] Demonstration of *Leishmania* parasites in bone marrow aspirate or in other biologic specimens, either by visualization or culture, is the most reliable diagnostic technique in the setting of HIV co-infection. However, invasive procedures require trained physicians and microscopic examination is time-consuming. Despite antileishmanial antibodies have high diagnostic value in immunocompetent patients,[6] serological tests are less reliable for immunosuppressed individuals.[7] There is some doubt whether one serological technique would be superior to the other for the VL diagnosis among HIV-infected patients and if there is difference in tests performance among global regions.[7] Indirect fluorescent antibody test (IFAT) remains the routine serological test used by the public health services in Brazil, despite requiring fluorescence microscopes and relatively well equipped laboratories. The direct agglutination test (DAT) offers high sensitivity and specificity and may be performed in laboratories with limited infrastructure. [8 9 10] Similarly, the development of the rapid recombinant K39 antigen-based immunochromatographic tests (rK39) has brought a major improvement in the diagnosis of VL in non HIV-infected patients in the field.[8] Nevertheless,

the paucity of data about these rapid tests in HIV-infected patients [11] [12] makes clear the need for more research before it being integrated in a diagnostic algorithm. In addition, in recent years, different molecular methods, particularly polymerase chain reaction (PCR), have successively been evaluated as a sensitive and specific alternative for the diagnosis of leishmaniasis[13] but its application outside Europe, in areas of high endemicity, is still poorly studied. The objective of the present study is to evaluate the diagnostic accuracy of spleen palpation, parasitological, molecular and serological tests for the diagnosis of VL among HIV-infected symptomatic patients in a reference center in Brazil.

PATIENTS AND METHODS

This study is part of a cohort involving *Leishmania*-HIV coinfected patients in progress in Belo Horizonte, Minas Gerais, in a reference center in Brazil, Eduardo de Menezes Hospital, Fundação Hospitalar do Estado de Minas Gerais (HEM-FHEMIG). Minas Gerais state has a population of around twenty million people. Patients with infectious diseases from the capital Belo Horizonte and from small cities of the state are referred by Brazilian universal health system, the "Sistema Único de Saúde". All patients with medical suspicion of VL were invited to participate. The study was planned as a delayed-type cross-sectional study where clinical follow up was used to enhance the validity of the reference standard (which we named target condition) defined by an adjudication committee. Patients were enrolled consecutively until the sample required had been reached.

Approval for this study was obtained from the Ethical Review Board of HEM-FHEMIG and from Centro de Pesquisas René Rachou (CPqRR), Fundação Oswaldo Cruz. Patients were included in the study only after appropriate informed consent was obtained. The flow diagram

which describes the design of the study and the flow of patients according to Standards for Reporting of Diagnostic Accuracy **(**STARD) statement [14] is presented in Figure 1.

Clinical suspicion for VL was defined as a history of more than 14 days of fever or splenomegaly or cytopenia. Cytopenia was defined as a hemoglobin level < 11 g/dL and/or a white blood cell count < 3.5 x 10^9/L and/or a platelet count < 120 × 10^9/L. Patients with clinical suspicion were eligible to participate only if they had not received any treatment for visceral leishmaniasis. A questionnaire with clinical, epidemiologic and demographic data was filled out for each patient at enrollment and diagnostic procedures were performed when consented: bone marrow aspiration and a venous blood sample were taken. Each serological technique, direct smear examination and culture of the bone marrow samples were carried out in separate services by different laboratory personnel who were not aware of results of the other tests. Regardless of the study or pending tests results, the decision to treat leishmaniasis was defined by hospital staff based on clinical and laboratory findings, and also on the results of the parallel investigation for other diseases.

Adjudication committee definition Given the lack of a gold standard for the diagnosis of VL it was formed an adjudication committee with 4 members (AR, GFC, BMFN, MRS) that decided by consensus, after clinical follow-up, about the presence or absence of the target condition under study (VL). This expert panel evaluated results of all tests available, including those performed for other diagnostic possibilities and outcomes observed at follow-up by reviewing the medical records four weeks after the last patient inclusion in the study. Patients were considered as having the target condition if clinical symptoms were judged being caused by *Leishmania (Leishmania) infantum*.

Direct examination Six good-quality smears prepared from bone marrow aspirate and stained with Leishman stain were examined under an oil immersion light microscope for 45 min each time. *Leishmania* spp. bodies in the smear were confirmed independently by two experienced microscopists by detection of the standard parasite morphology: a typical oval or elliptical cells, bounded by a cytoplasmic membrane containing the nucleus and kinetoplast.

***Leishmania* culture** The material of bone marrow aspirate (BMA) was immediately placed in culture tubes containing biphasic medium NNN (McNeal, Novy & Nicolle) and 500 µL of LIT (Liver Infusion Tryptose) supplemented with 20% heat-inactivated fetal bovine serum (FBS; GIBCO/Invitrogen, Grand Island, NY, US) plus penicillin (50U/mL) and streptomycin (50 µ/ml). The cultures were incubated at 26°C, and weekly examined, by microscopy (400× magnification) for the presence of promastigote of *Leishmania* spp. for a total of 30 days.

Real time PCR Total DNA from peripheral blood of patients was extracted using the QIAamp DNA Blood mini-kit (Qiagen GMbH; Hilden, DE). Two independent assays for the detection and quantification of *Leishmania* spp. and human DNA were performed using the StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, US). For the *Leishmania* assay, the target DNA was the small-subunit ribosomal RNA (SSU rRNA) gene, which is conserved among all *Leishmania* species. It consisted of the primers LEIS.U1 (5'-AAGTGCTTTCCCATCGCAACT-3') and LEIS.L1 (5'-GACGCACTAAACCCCCTCCAA-3'), designed to amplify a 67-bp fragment and the fluorogenic probe LEIS.P1 (FAM 5'-CGGTTCGGTGTGTGGCGCC-3'TAMRA), as described by Wortmann (2002).[15] The protocol described by Gomes (2012)[16] was applied. For the human assay, the *ACTB* reference gene was used as target and the primers Aco1 and Aco2, [17] which generate 120-bp fragments, were used in this assay. The reaction mixtures contained 12.5 µL of Syber® Green PCR

Master Mix 2X (Life Technologies), 0.1 μM of each primer, and 3 μL of DNA template in a final volume of 25 μL. The cycling parameters were universal, and the melting analysis was conducted based on the parameters of the StepOnePlus™ Real-Time PCR System. Standard curves were prepared for each assay using known quantities of pCR-4 TOPO vector (Life Technologies) containing the cloned human gene actin, beta (ACTB; 120 bp) and the 67 bp *L. infantum* SSU rRNA fragment. The recombinant plasmids were serially diluted 1:10 to create each standard curve. The quality parameters of the standard curves, including PCR efficiency, linear dynamic range and correlation coefficient, were obtained by software analysis and were accurate and similar to obtained by previous studies from our group.[16 18] The parasite load was expressed by the *Leishmania* DNA load (relative copy number of the 67 bp SSU rRNA fragment) normalized against the reference gene *ACTB*, according to Overbergh, 1999.[19] *ACTB* copy numbers for the target samples were divided by the highest *ACTB* value obtained in the experiment, resulting in a correction factor used for normalization.

Serological tests The presence of *L. infantum*-specific antibodies was determined by three different methods: a rapid rK39 antigen-based immunochromatographic test dipstick test (Kalazar Detect®), IFAT (IFI®, Bio-Manguinhos®) and a direct agglutination test using a prototype kit produced at Laboratório de Pesquisas Clínicas of CPqRR (DAT-LPC). The dipstick test was performed according to the manufacturers' instructions (InBios International, Inc. Seattle, WA, US). Twenty microliters of serum were mixed with two drops of buffer provided with the test and placed on a cellulose strip. Following the manufacturer's instructions, a test result was positive when two bands, a control band, and a positive test band appeared within 10 min. The test result was negative only if the control band appeared. The IFAT was carried out at the Parasitic Diseases Laboratory of the Fundação Ezequiel Dias, using cultured promastigotes of *Leishmania major* as antigen. A cut-off value 1:80 was used

to establish a positive IFAT result. DAT-LPC was produced using freeze-dried antigen developed with *Leishmania (L.) infantum* (MHOM/BR/2002/LPC-RPV) and prepared as described by El Harith[20] following the improvements recommended by Oliveira. [21] Firstly, the 10X concentrated physiologic solution (9% NaCl plus 1% sodium azide) was diluted 1:10 with type I water and 5 ml were added into antigen vial, which was carefully homogenized. Next, the 10 X concentrated diluents solution (9% NaCl; 5 mM N-Acetyl Cystein (NAC); 1% sodium azide) was diluted 1:10 with physiologic solution (0,9% NaCl). Sera were diluted in diluents solution and a two-fold dilution series was made from 1:100 to 1:102,400. Then, 50 µl of DAT-LPC antigen suspension (concentration 2×10^7 parasites/ml) was added to each well of a V-shaped microtitre plate (Greiner Bio-One, Americana, SP, Brazil) containing 50 µl of diluted serum. After a minimum incubation of 4 h at room temperature, the end titre was read as the dilution immediately before the well with a clear sharp-edged blue spot identical in size to the negative control.

Statistical analysis In order to estimate a sample size we used the following strategy. Since IFAT is still widely used in Brazil, there is interest in its comparison with DAT. The software MedCalc® version 9.4.2.0 [22, 23] calculated a sample size of 47 patients for the comparison of the areas under two ROC curves (DAT and IFAT tests as continuous variable, derived from the same cases). This estimation of sample size takes into account the significance level of 0.05 and power of 0.80, considering the hypothesized areas for IFAT and DAT ROC curves of 0.78 and 0.92,[7] respectively. The software used requires hypothesized rank correlation coefficient in the positive group (abnormal cases) and in the negative cases (normal cases). We estimated these values respectively 0.46 and 0.05, a conservative estimation derived from a veterinary study. [24] If we maintain all parameters and change correlation coefficients in the positive and negative groups to 0.81 and 0.05, respectively, an estimation derived from a

human study, [9] sample size needed would be 49 patients. Another comparison of interest is DAT versus PCR. The software calculated the required sample size of 99 patients for the comparison of the areas under DAT and PCR ROC curves [22, 23] derived from same cases. This estimate takes into account the significance level of 0.05 and power of 0.80, considering the hypothesize area for DAT and PCR ROC curves of 0.92 and 0.98, respectively.[7] The hypothesized rank correlation coefficient in the positive and negative groups were estimated 0.5 and 0.5, a conservative estimates from Deborggraeve and collaborators, 2008. [25] Finally, a sample size of roughly one hundred patients is needed for latent class analysis.[26] Descriptive statistical analysis was performed in MedCalc® software version 9.4.2.0. Categorical variables were analyzed by chi-squared or Fisher exact tests. The distribution of continuous variables was compared by the Mann-Whitney test. Continuous variables were described by mean and its standard deviation or median with interquartile range (IR 25-75%). ANOVA with Levene's test for equality of variances was used for continuous variables with parametric distribution. Wilcoxon rank-sum test was performed for nonparametric variables. Sensitivity and specificity of index tests were estimated using two reference comparators: parasitological test and adjudication committee final diagnosis. The tests' performances were also estimated through latent class model (LCM) analyses obtained by using R Program Software. Latent class analysis is a mathematical modeling technique based on the idea that the true disease status for each patient is unknown and needs to be estimated from the data. It can be thought as the analogue of factor analysis for categorical data. LCM attempts to model associations between observed categorical variables by assuming that a non-observed (latent) variable is determining these associations. In diagnostic test validation, the true disease status of an individual can be considered as a dichotomous latent variable with two categories, "infected" and "not infected". Within a group of individuals with unknown disease status, for whom at least three independent diagnostic test results are available, LCM will model the probability

of each combination of test results (or response pattern) conditional on the latent class. An estimate of both disease prevalence and sensitivity and specificity of all tests can be derived from the pattern of diagnostic test results as expected under the latent class model. [26]

The sensitivity and specificity of different cutoff values of IFAT and DAT in predicting VL were determined by the construction of a receiver operating characteristic curves (ROC curve). The area under curve (AUC) obtained from the ROC curve was used as a measure of global accuracy.

RESULTS

One hundred and seventy-eight patients in which one of the differential diagnoses could be visceral leishmaniasis were evaluated over a period of 2 years (from March, 2011 to February, 2013). Out of this total, 115 were carriers of HIV. Two cases were excluded from the analysis: one did not fulfill the suspicion criteria and another patient because of amphotericin B use before the diagnostic evaluation. The clinical characteristics of the 113 included patients are presented in Table 1. Twenty four patients (21.2%) had one or more VL episodes in the past, sixty seven (59%) had present or past opportunistic infection and 70 patients (62%) were taking antiretroviral (ARV) therapy at study inclusion.

The diagnosis of VL was reached by parasitological confirmation in 41 of the 113 patients: 38 (92.7%) by direct examination and 3 patients (7.3%) by culture of bone marrow specimen. Two patients did not allow the bone marrow aspiration for parasite examination. Besides these 41 patients, six other cases were treated for leishmaniasis, including these two cases that have not undergone the sample aspiration of bone marrow, what totalized 47 treated as VL by

the hospital staff. Five of these six patients were also considered by the adjudication committee as true VL-cases (46 out 113 VL-suspect patients) on the basis of the overall case clinical picture, available tests, therapeutic response and clinical follow-up data (details about these patients including clinical picture, outcomes and test results are shown in supplementary table). By having the adjudication committee diagnosis as the reference test, the sensitivity and specificity of parasitological exam were respectively 93.2 % (CI95% 81.3-98.5) and 100 % (CI95% 94.6-100).

There were 72 VL suspect patients with a negative parasitology and out them, 59 (82%) had at least one clinical assessment after 4 weeks of the study enrollment. The median follow-up time by reviewing the medical records was 38 weeks, ranging from 7 to 94 weeks. Through clinical follow-up an alternative diagnosis (non-VL) was confirmed in 19 (26.4%) patients by a histological, microbiological or laboratorial exam. For 35 (48.6%) patients, the alternative diagnosis was deemed most likely on clinical grounds. Nine cases (12.5%) remained without a conclusive diagnosis. The adjudication committee examined all parasitological negative cases and the target condition (VL) was considered present in five out 72 patients.

The characteristics of VL patients with and without positive parasitological examination were compared and it was found no statistical difference in clinical and laboratory variables (data not shown). The clinical and laboratory characteristics of VL and non-VL cases according to adjudication committee definition are shown in Table 2. The most important clinical differences observed between the two groups were a higher splenomegaly rate (76% x 22%) and a lower leucocyte count median in VL group. The 30-day mortality also differed between patients with and without VL diagnosis, significantly higher in non-VL patients. It is probably related to the most often diagnosis in the non-VL group: disseminated tuberculosis, a very

common condition reported in studies evaluating hospitalized HIV-infected patients, a disease with high mortality rate.[27] [28] The quantification of the parasite load by qPCR exhibited great variability in each group and there was no statistical difference between VL and non-VL patients. It should be emphasized that these results were obtained from only four qPCR-positive patients in the control group, which prevents the extrapolation of these findings.

Sensitivity, specificity and 95% confidence intervals (CI 95%) of index tests were calculated using conventional formulas and having the target condition or parasitological results as reference test or through LCM (Table 3). In all three analyses, rK39 dipstick test showed the lowest sensitivity and highest specificity. There was no statistical difference among performance presented by DAT-LPC and qPCR. The IFAT exhibited a poor performance as demonstrated by sensitivity below 65% independently of reference test used.

Figure 2 presents the performance of the DAT-LPC and IFAT in diagnosis of VL in HIV-infected patients using adjudication committee definition as reference test. The accuracy of DAT-LPC based on the area under the ROC curve was 0.94 (Standard error 0,026; CI 95% 0.87-0.97; p=0.0001), which means good overall performance. In turn, performance exhibited by IFAT was significantly lower (p=0.006), with global measure of accuracy by AUC of 0.82 (Standard error 0.043; CI 95% 0.73-0.89), p=0.001).

DISCUSSION

To our knowledge, no study so far in the Americas has addressed VL diagnostics by a comparative assessment of serological and molecular tests in HIV-infected patients. Strengths

of this study are that it includes symptomatic patients living in an endemic area and its delayed-type cross sectional design.

The population studied here presents an unexpected predominance of women, different from other published series. We have no clear explanation for this yet. Although both AIDS and VL predominantly affect men, it has recently changed. The spread of AIDS among women has been observed in almost all continents, and these account for 48% of cases worldwide. [29] In Brazil, the sex ratio clearly shows this trend: in 1986, there were 15:1 male to female case and, in 2009, this ratio was 1.6:1. [30] Also may have contributed the recent urban behavior of VL, since predominance of men over women in exposure to *Leishmania* spp. caused by the labor activity in the field no longer exists. Regarding to the clinical aspects, the high prevalence of palpable spleen and severe leukopenia among true VL-cases are the most significant aspects that may increase clinical suspicion of VL.

A widely documented problem hampering diagnosis research in VL is the absence of a true gold standard to classify with certainty patients as having the target condition or not. Thus, the published sensitivity and specificity estimates of tests might be biased to some extent, depending on the controls and reference test used. Microscopic examination and culture of bone marrow, lymph node, and spleen aspiration are the conventional diagnostic procedures. Parasitology for VL is very specific, but unless spleen aspirates can be taken, its sensitivity is less than 90%. [31] When a reference test with sub-optimal sensitivity for case ascertainment is used, true VL cases are missed and therefore included in the group of controls. They will generate a "false-positive" result in any new test one wishes to evaluate (assuming this new test is 100% sensitive). For those cases, the new test is actually right while the reference test is wrong, and the specificity of the new test will thus be systematically underestimated [32]. For

these problems, the term ‘reference standard’ has been introduced. This term acknowledges the absence of a ‘gold standard’ and refers to the best available method for classifying patients as having the target condition. Consensus diagnosis definition by an adjudication committee using follow-up information is an attractive alternative if a generally accepted reference standard does not exist and multiple sources of information have to be interpreted in a judicious way to reach a diagnosis.[33] Another strategy used to deal with the lack of a perfect gold standard was LCM analysis.

Parasitological test proved to be the most sensitive test for diagnosing VL in HIV-infected patients in our series. The sensitivity of direct examination performed on slides from bone marrow aspirate in our study was higher than the 70% reported in immunocompetent patients.[31] This observation can strengthen the hypothesis of higher *Leishmania* spp. parasitemia in HIV-infected patients, as already suggested by other authors.[34] [13] Although this fact legitimizes the test as a good diagnostic strategy, obtaining the material requires invasive and painful technique. Furthermore, several factors interfere with the positivity rates of the method. Bone marrow aspiration should be carried out by trained individuals, and performance of a correct technical procedure is vital to its sensitivity. Finally, the expertise and persistence of the microscopist are also factors of utmost importance influencing the final performance of the test.

Serologic tests have a high diagnostic value for VL diagnosis in immunocompetent patients [35] [36] but their value is limited in HIV-infected patients.[7] Our serology results confirm the low sensitivity of all tests except DAT-LPC for VL diagnosis. Although rk39 dipstick has an ideal format for use in the field, as it is a rapid and simple test not requiring extensive training of the operator, its lower sensitivity limits its use for VL diagnosis among HIV-infected patients.

Unsatisfactory results also have been observed in Africa [8] [37] and one possible explanation would be the high prevalence of HIV co-infection, often unrecognized.

DAT is a well validated test for serodiagnosis of VL and has been used for the last two decade in immunocompetent individuals, combining high levels of intrinsic validity and ease of use.[8,] [38] In the present study, DAT-LPC was highly sensitive and was found to be a suitable alternative to screening visceral leishmaniasis in HIV infected patients. Of the 46 patients considered VL-cases by adjudication committee, 41 were identified by DAT-LPC (89%). Out from five unidentified VL-cases, in four the diagnosis could be done by both parasitological examination and blood qPCR-*Leishmania*. The ability to make most VL diagnoses without the need for specialized equipment or invasive procedures makes this test a major advance for peripheral health care facilities. Eight patients had a positive DAT-LPC and were considered non VL-cases by adjudication committee: in two qPCR-*Leishmania* was also positive in peripheral blood and another had presented VL years before and was under prophylactic use of amphotericin B. Despite the target condition was also considered absent in this patient, he had chronic abnormalities such as thrombocytopenia and splenomegaly, consistent with a recently recognized form of VL among HIV infected patients: a chronic active form. [39] These observations suggest that some of patients with considered false-positive results of DAT-LPC were truly infected with *Leishmania* spp., although *Leishmania* was not the etiologic agent of the disease under investigation. A fourth patient was empirically treated for VL and disseminated mycobacteriosis with no therapeutic response. This patient had very advanced immunosuppression. He discontinued the use of all medications and died in a few months without confirmed etiological diagnosis. As suggested by others, [36] the specificity of the DAT, like any other diagnostic test, depends to a certain extent on the choice of the control group. When control groups are composed of non-symptomatic people or patients with other

confirmed diseases, the specificity of the test tends to be higher than when only clinical suspects are studied, as is the case here.

Molecular diagnosis exploiting PCR combines several advantages; it is minimally invasive, has a high sensitivity and specificity and is capable of identifying relapses and reinfections in treated VL patients. The sensitivity exhibited by qPCR (based on SSUrRNA target and performed on blood samples) not overcame but it was as good as the parasitological test. This is beneficial because the use of a test with blood means that invasive diagnostic procedures can be avoided. Previous studies with HIV-infected patients have demonstrated that the sensitivity of PCR for the diagnosis of VL ranges from 76 to 100%.[13 39 34 40 41 42] However, two points need to be noted: first, PCR is not one technique but a method encompassing a number of techniques depending on a variety of factors. Therefore, a direct comparison between results from studies using different PCR should not be made. The choice of different PCR targets should be guided by the aim to which it is directed. In fact, the PCR assay based on kinetoplast amplification is probably the most sensitive because this molecular target is present in about 10,000 copies per parasite.[43] However, the heterogeneity of kinetoplast minicircles can be a problem for accurate quantification.[13] Moreover, the high sensitivity of the kinetoplast target might be a double-sword edge when used as a tool to verify the therapeutic response. For that reason, we chose the small subunit rRNA gene of the parasite as the target sequence. The goal of our group was to evaluate a test that could be applied not only in diagnosis but also in monitoring of HIV-infected patients after VL treatment (there is a cohort in progress). The other point to note is that the estimates of sensitivity and specificity should be carefully interpreted according to sample selection criterion. Studies using sera from healthy controls in non-endemic areas or from patients with other confirmed infectious diseases might overestimate the specificity of the test. In our series 6% qPCR positivity (4

out 67 patients) in control group was observed. Two out them were treated for other conditions (one patient had pulmonary tuberculosis and another had secondary syphilis associated with vitamin B12 deficiency) and evolved with complete remission of fever, splenomegaly and cytopenia. The third patient had VL years before. After the initial study evaluation he had complete improvement of cytopenia with the reintroduction of antiretroviral therapy that he had interrupted. The favorable evolution without specific treatment for VL suggests that these three patients were asymptomatic carriers of *Leishmania* spp. The fourth patient had disseminated cryptococcosis and died in few days due to neurological complications. In this case, it was not possible undoubtedly attest that *Leishmania* spp. infection was not liable for some of his symptoms.

In conclusion, our results show that, in an urban setting in Brazil, unlike other serological techniques, DAT is a suitable tool for the VL diagnosis among HIV-infected patients. DAT is a simple, inexpensive technique with reasonable specificity and sensitivity. It uses very little serum, can use plasma as well, and is performed at room temperature. Nevertheless, the laboratory infrastructure needed to carry out DAT is much simpler than IFAT, as no sophisticated equipment is required. We reiterate that DAT rather than rk39 strip test or IFAT can be routinely applied for VL diagnosis in HIV-infected patients.

Despite of high sensitivity and specificity of the qPCR based on SSUrRNA target, qPCR diagnosis may have limitations in VL diagnosis. Real time PCR diagnosis alone can also not differentiate between asymptomatic and symptomatic VL-infections. Thus this method would be used only for the confirmation of suspected cases of active disease, excluded others diagnoses.

No currently available method for VL diagnosis exhibits all the desirable characteristics of high sensitivity and specificity, in addition to ease of use and low cost. This emphasizes the importance of associating serology, parasitological and molecular methods in order to reach higher positivity rates in diagnosis. Using DAT as screening test, followed by parasitological tests only in DAT-negative cases, confirmation of VL might be possible in nearly 100% and invasive tests will be done in no more than 11% of the patients. Molecular tests, including based on SSUrRNA target and performed in peripheral blood, represent a non-invasive alternative for VL confirmation in settings with adequate laboratory infrastructure.

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Ethical approval The Ethical Review Boards of Centro de Pesquisas René Rachou (CPqRR-FIOCRUZ) and of Hospital Eduardo de Menezes (HEM-FHEMIG), Belo Horizonte, MG, Brazil, approved the study in agreement with Resolution 357/05 of the National Health Council of the Ministry of Health, which regulates research involving human subjects in Brazil.

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Table 1. Demographic and clinical characteristics of 113 HIV-infected patients who underwent diagnostic testing for VL

Variable	**Patients**
Sex (male: female)	39:74
Age, mean years + SD	40,42 ± 10.22
Previous VL (%)	24/113 (21.2%)
CD4 count, median (25-75% interquartile range) *	67 (37-164) cell/mm^3
PCR-HIV-1 load, median (25-75% interquartile range) &	34.176 (79-184.370) copies/mm^3
ARV therapy use (%)	70/113 (61.9%)
Fever more than 14 days (%)	81/113 (71.7%)
Cytopenia (%)	111/113 (98.2%)
Splenomegaly (%)	50/113 (44.2%)
Opportunist infection previously (%)	67/113 (59.3%)

Abbreviations: **SD**: standard deviation **VL**: visceral leishmaniasis **PCR-HIV-1**: HIV load by real time polymerase chain reaction **ARV**: anti-retroviral * available in 95 patients & available in 69 patients

Table 2. Clinical and laboratorial characteristics of patients with target condition (visceral leishmaniasis) present or absent according to adjudication committee definition

	Target condition present n=46	**Target condition absent n=67**	**p value**
Age (mean ± SD)	41.02 ± 10.8	40.0 ± 9.8	0.60
Sex (male: female)	11:35	28:39	0.07
Anti-HVC presence	2 (4.3%)	6 (9.5%)	0,44
HbsAg presence	0	2 (3.2%)	0.31
Illicit drug use	16 (34.8%)	27 (40.3 %)	0.58
Alcohol abuse	31 (67.4%)	40 (59.7%)	0.39
Previous VL diagnosis	20 (43.4%)	4 (5.9%)	0.00
Previous opportunist infection	31 (67.4%)	36 (53.7%)	0.17
Previous schistosomiasis diagnosis	3 (6.5%)	7 (10.4 %)	0.05
Comorbidities	13 (28.3%)	16 (23.8%)	0.64
CD4+ T cell count (25-75% IR)	67 (6-864)[a]	92 (5-483)[b]	0.18
HIV-PCR load copies/mm^3 (25-75% IR)	5.000 (0-65.712)[c]	78.132 (7.412-316.650)[d]	0.01
Fever	28 (60.8%)	53 (79.1 %)	0.06
Cytopenia	46 (100%)	65 (97.0 %)	0.51
Splenomegaly on physical exam	35 (76%)	15 (22.4 %)	0.00
Death in 30 days	4 (8.7%)	8 (11.9%)	0.02
ARV therapy use	32 (69.6%)	34 (50.7)	0.15
ARV therapy regular use	15/32 (46.8%)	7/34 (20.6)	0.01
Hemoglobin g/dl	8.2 ± 1.6	9.1 ± 2.4	0.04
Leucocytes count cell/L (25-75% IR)	2.0 (1.75-2.80) × 10^9	3.3 (1.75-4.20) × 10^9	0.005
Platelets count cell/L (25-75% IR)	114 (82.75 -173.75) ×10^9	139 (90.50-244.50) ×10^9	0.233
rK39 dipstick test positivity	21/46 (45.6%)	2/67 (3.0%)	0.00
IFAT positivity	28/46 (60.9%)	7/67 (10.4%)	0.00
DAT-LPC positivity	41/46 (89%)	8/59 (13.6%)	0.00
***Leishmania* qPCR in peripheral blood positivity**	36/42 (85.7%)	4/60 (6.7%)	0.00
Median copies *Leishmania* qPCR in blood (25-75% IR)	16.543 (108-70.209) [e]	66.436 (5066-36.479.000)[f]	0.56

Abbreviations**: SD**: standard deviation **Anti-HVC:** antibody to the hepatitis C virus **HbsAg**: hepatitis B surface antigen **ARV:** anti-retroviral **PCR-HIV:** real time polymerase chain reaction for HIV-1 **rK39 dipstick test:** rapid recombinant K39 antigen-based immunochromatographic test **IFAT:** indirect fluorescent antibody test **DAT-LPC:** a direct agglutination test using a prototype kit produced at CPqRR **qPCR:** real time polymerase chain reaction for *Leishmania infantum* **a:** performed in 43 patients **b**: performed in 52 patients **c**: performed in 29 patients **d:** performed in 40 patients **e**: available in 35 patients **f**: available in 4 patients

Table 3. Sensitivity, specficity and 95% confidence intervals (95% CI) presented by index tests using two reference tests and Latent Class Model analysis

	Parasitological test as reference test		**Adjudication committee diagnosis as reference test**		**Latent class model analyses**	
Index test	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
rK39 dipstick test (Kalazar Detect™®)	46.6 (30.7-62.6)	97.1 (90.0-99.6)	45.6 (30.9-61.0)	97.0 (89.6-99.5)	46.2 (31.3-61.7)	98.4 (89.3-99.8)
IFAT	61.0 (44.5-75.8)	87.1 (77.0-94.0)	60.9 (45.4-74.9)	89.5 (79.6-95.7)	61.5 (45.6-75.3)	88.5 (77.8-94.4)
DAT-LPC	87.8 (73.8-95.9)	82.3 (70.5-90.8)	89.1 (76.4-96.3)	86.4 (75.0-93.9)	89.7 (75.7-96.1)	85.3 (74.0-92.1)
qPCR in peripheral blood	87.2 (72.6-95.7)	93.4 (84.0-98.1)	85.7 (71.0.-94.5)	94.9 (85.8-98.9)	84.6 (69.7-92.9)	91.8 (81.8-96.6)
Splenomegaly on physical exam	75.6 (59.7-87.6)	79.4 (67.4-88.3)	76.1 (61.2-87.4)	81.5 (70.0-90.1)	79.5 (64.0-89.4)	80.3 (68.5-88.5)

rK39 dipstick test: rapid recombinant K39 antigen-based immunochromatographic test **IFAT:** indirect fluorescent antibody test **DAT-LPC:** a direct agglutination test using a prototype kit produced at CPqRR **qPCR:** real time polymerase chain reaction for *Leishmania infantum*

Figure 1.

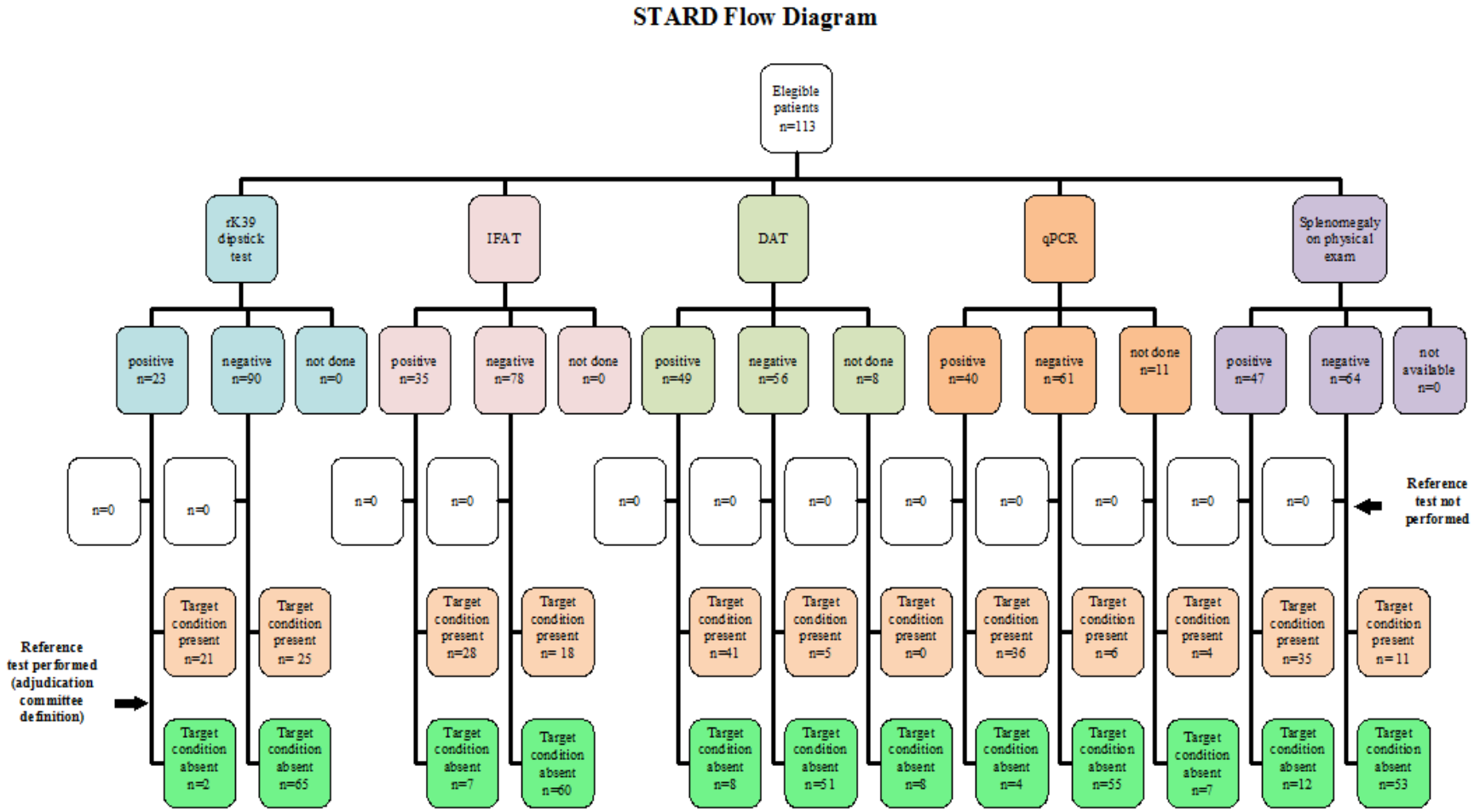
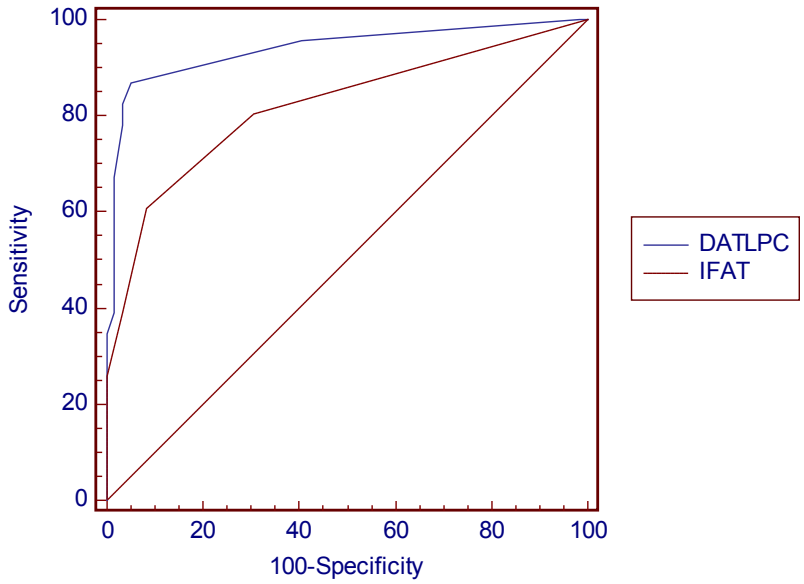

STARD Flow Diagram
Elegible patients n=113
rK39 dipstick test
IFAT
DAT
qPCR
Splenomegaly on physical exam
positive n=23
negative n=90
not done n=0
positive n=35
negative n=78
not done n=0
positive n=49
negative n=56
not done n=8
positive n=40
negative n=61
not done n=11
positive n=47
negative n=64
not available n=0
n=0
Reference test not performed
Reference test performed (adjudication committee definition)
Target condition present n=21
Target condition present n= 25
Target condition present n=28
Target condition present n= 18
Target condition present n=41
Target condition present n=5
Target condition present n=0
Target condition present n=36
Target condition present n=6
Target condition present n=4
Target condition present n=35
Target condition present n= 11
Target condition absent n=2
Target condition absent n=65
Target condition absent n=7
Target condition absent n=60
Target condition absent n=8
Target condition absent n=51
Target condition absent n=8
Target condition absent n=4
Target condition absent n=55
Target condition absent n=7
Target condition absent n=12
Target condition absent n=53

Figure 2. Receiver operating characteristic curve for DAT-LPC and IFAT



Footnote: Pairwise comparison of ROC curve: difference between areas 0,120, Standard error 0.0434, 95% Confidence interval 0.0345 to 0.205, Significance level

P = 0.006

STARD checklist for reporting of studies of diagnostic accuracy

(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	(3)
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	(4)
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	(4, 5, 6)
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	recruitment based on presence of symptoms (5)
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	consecutive series of participants defined by presence of symptoms (6)
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	data collection planned before the index test (prospective study)(5)
Test methods	7	The reference standard and its rationale.	two reference standard were compared (5)
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	(6-8)
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	(6-8)
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	(6-8)
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	(5)
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	(9)
	13	Methods for calculating test reproducibility, if done.	Not done
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	(9)
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	Table 1
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	Figure 1
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	All test were performed simultaneously before treatment
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	Table 2
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	Table 2 and Figure 2
	20	Any adverse events from performing the index tests or the reference standard.	NA
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	Table 3
	22	How indeterminate results, missing data and outliers of the index tests were handled.	Table 2 and Figure 1
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	NA
	24	Estimates of test reproducibility, if done.	NA
DISCUSSION	25	Discuss the clinical applicability of the study findings.	(11-14)

5 CONSIDERAÇÕES FINAIS

Nos últimos 10 anos, várias descobertas científicas foram alcançadas no diagnóstico, terapia e prevenção das leishmanioses, e os preços de algumas das medicações usadas no tratamento foram reduzidos [2, 98]. Estes avanços, embora tenham facilitado a implantação de programas de controle, não foram suficientes para multiplicar estas iniciativas e causar impacto na mortalidade e na morbidade da doença, em todo o mundo [4].

Um marco estratégico foi alcançado em 2007, quando a Assembleia Mundial da Saúde aprovou a resolução número 60 para o controle da leishmaniose [12], que definiu diretrizes para orientar pesquisas e programas de prevenção. Foram feitas recomendações sobre o uso de testes diagnósticos, sobre o manejo da coinfecção *Leishmania-HIV* e considerações sobre a influência de fatores sociais e mudanças climáticas na disseminação da doença. Identificou-se a clara necessidade de fortalecimento, tanto da detecção de casos ativos de leishmaniose quanto da capacidade diagnóstica em unidade de saúde periférica, onde os pacientes são rotineiramente tratados com base em diagnóstico presuntivo. Recomendações para a pesquisa incluíram a promoção do conhecimento epidemiológico desta parasitose e avaliação de custo efetividade dos meios diagnósticos e terapêuticos.

Até agora, a confirmação diagnóstica de LV baseou-se na identificação do parasito por microscopia. A maioria dos serviços, no entanto, não dispõe dos recursos necessários para a propedêutica invasiva. Outras alternativas diagnósticas foram introduzidas nos últimos anos, incluindo técnicas sorológicas e moleculares, mas no caso dos pacientes coinfectados pelo HIV, a evidência disponível sobre essas ferramentas diagnósticas, além de escassa, não é robusta o suficiente para embasar as decisões clínicas. Da mesma forma, faltam trabalhos bem desenhados capazes de identificar os fatores preditores do curso recorrente após o tratamento para LV, que auxiliariam na decisão de introduzir e suspender a profilaxia secundária, estratégia que expõe o paciente a risco e onera o sistema de saúde.

Revisando a literatura de forma sistemática foi possível compilar e comparar o desempenho dos vários métodos sorológicos e moleculares disponíveis, o que permitiu a identificação de suas principais propriedades e, consequentemente, reflexão sobre as implicações do uso de cada um deles. Exceto para o DAT, cuja

experiência entre infectados pelo HIV concentra-se principalmente na Índia (portanto, com *L.* (L.) *donovani*), para os outros testes sorológicos as evidências foram produzidas principalmente na Europa e, portanto, referem-se essencialmente à infecção por *L.(L.) infantum*. Com base nas evidências até agora disponíveis, testes baseados na detecção de DNA são altamente sensíveis e apresentaram desempenho global elevado, podendo contribuir para a confirmação diagnóstica. No estudo clínico que compõe essa tese (**ARTIGO 4**), o desempenho dos principais testes em nosso meio foi avaliado utilizando-se uma amostra calculada para tal fim e sob condições adequadas para um estudo de acurácia: os testes foram aplicados em todos os suspeitos, casos e controles, de forma "mascarada" e simultânea. Até onde sabemos, esse trabalho representa o primeiro estudo de validação diagnóstica para LV com desenho transversal, incluindo dados de seguimento, e o primeiro estudo planejado para comparar os diversos testes diagnósticos para LV entre infectados pelo HIV produzido nas Américas. Os resultados obtidos sugerem que os testes sorológicos não devem ser usados para descartar o diagnóstico de VL entre infectados pelo HIV. Observamos diferença de desempenho entre as várias técnicas sorológicas e superioridade do DAT em relação às demais. O desempenho do DAT observado em nosso meio, com *L.(L.) infantum*, se assemelha ao já descrito na Índia com *L.(L.) donovani*. A técnica de qPCR, utilizando a subunidade ribossomal do RNA como alvo, mostrou-se sensível e específica, despontando como alternativa ao exame parasitológico invasivo para a confirmação de casos suspeitos. Em relação à predição de recidiva, as evidências reunidas na literatura indicam a necessidade de instituição da profilaxia secundária após o tratamento de LV entre infectados pelo HIV, especialmente na presença de condições que foram identificadas como relacionadas a maior risco de recidiva, a saber: a ausência de elevação na contagem de linfócitos T CD4+ no seguimento, história anterior de recidiva de LV e contagem de linfócitos T CD4+ inferior a 100 células/dL na ocasião do diagnóstico primário de LV. Ao contrário do observado com outras infecções oportunistas, o uso de terapia antirretroviral altamente potente se mostrou insuficiente para evitar as recidivas de LV. Também com base em informações publicadas, o tratamento com derivados do antimônio se associa a uma mortalidade três vezes superior à observada com anfotericina B. Não há evidência suficiente até agora que indique superioridade de uma formulação de anfotericina sobre as demais, tampouco a dose ideal necessária deste medicamento. Estas conclusões trazem alguma luz sobre questões frequentes

no manejo clínico de pacientes coinfectados por *Leishmania*-HIV e devem contribuir para o estabelecimento de recomendações e rotinas de diagnóstico e acompanhamento destes pacientes, além de auxiliarem no delineamento de grandes estudos prospectivos que ainda são necessários.

5.1 **IMPLICAÇÕES PARA A PRÁTICA**

A introdução da LV nas grandes cidades e a sua associação com a infecção pelo HIV configura realidade epidemiológica diversa daquela previamente conhecida, requerendo além de nova racionalidade para os sistemas de vigilância e de controle, abordagem clínica diferenciada e cheia de especificidades. Enfrentar esse desafio exige o conhecimento de toda experiência acumulada até aqui, além de investimento humano e material em novas estratégias de abordagem.

A aplicação dos resultados deste trabalho na prática clínica requer análise criteriosa de suas limitações. Nenhum trabalho das Américas e, no geral, poucos estudos provenientes do continente africano e Índia puderam ser incluídos nas revisões da literatura realizadas. Os estudos reunidos para a avaliação prognóstica e para a análise da acurácia diagnóstica dos vários métodos disponíveis eram, no geral, relatos da experiência dos serviços e não estudos com planejamento adequado. Da mesma forma, faltam ensaios randomizados e controlados que permitam comparações diretas entres os vários tratamentos atualmente disponíveis para LV. Apesar destas limitações, decisões clínicas precisam ser tomadas todos os dias. Nossas conclusões devem contribuir para embasar algumas dessas decisões, especialmente nos continentes europeu e sul-americano, em algumas situações descritas a seguir.

Sobre o diagnóstico de LV entre infectados pelo HIV:
1- os testes sorológicos para leishmaniose IFAT, ELISA, BLOT e DAT exibem baixa sensibilidade e não podem ser usados para afastar o diagnóstico de LV;
2- DAT exibe medida de desempenho global superior a apresentada por ELISA e IFAT e, sob o aspecto de acurácia, deve ser preferido em rotinas de investigação;

3- Incluindo nossos próprios resultados (**ARTIGO 4**), foram identificados apenas três estudos avaliando o desempenho de testes imunocromatográficos rápidos baseados na pesquisa do anticorpo contra o antígeno recombinante k39 entre infectados pelo HIV, sendo observada sensibilidade muito baixa desta técnica;
4- a técnica de PCR apresenta alto desempenho global e pode ser utilizada para confirmar ou afastar o diagnóstico de LV entre pacientes infectados pelo HIV com quadro clínico compatível, representando alternativa ao exame parasitológico para a confirmação diagnóstica;
5- o PCR em tempo real utilizando como alvo a subunidade ribossomal do RNA e testado em nosso meio mostrou-se técnica sensível e específica para o diagnóstico de LV.

Sobre a recidiva de LV entre infectados pelo HIV:
1- a terapia antirretroviral altamente potente não é intervenção suficiente para evitar a recidiva de LV;
2- a profilaxia secundária após o tratamento reduz significativamente a taxa de recidiva de LV e deve ser recomendada;
3- algumas condições, se identificadas, devem ser consideradas marcadoras do risco de recorrência de LV e reforçam a indicação de profilaxia secundária: ausência de elevação da contagem de linfócitos T CD4+ no seguimento; história prévia de recidiva de LV; contagem de linfócitos T CD4+ abaixo de 100 células/mL na ocasião do primeiro diagnóstico de LV.

Sobre o tratamento de LV entre infectados pelo HIV:
1- os derivados de antimônio são drogas mal toleradas pelos pacientes coinfectados pelo HIV, associando-se com mortalidade três vezes maior que a observada com o tratamento com anfotericina B e devem ser desaconselhadas;
2- os dados disponíveis até o momento são insuficientes para se comparar a eficácia entre as várias formulações de anfotericina B ou se definir a dose e tempo de tratamento ideais;
3- a experiência com miltefosina é limitada entre infectados pelo HIV.

5.2 IMPLICAÇÕES PARA A PESQUISA

Muitas questões permanecem não respondidas com relação à coinfecção *Leishmania*-HIV. Mais estudos seriam necessários para determinar se há diferença entre os vários esquemas profiláticos na redução de recidiva e qual o limiar de linfócitos T CD4+ que permitiria a suspensão da profilaxia.

A aplicação de metodologia rigorosa é muito importante no desenho e condução de estudos para avaliação de acurácia diagnóstica. Esta revisão apontou problemas principalmente relacionados às técnicas de seleção de amostra e não uniformização de critérios diagnósticos. Seria interessante que estudos futuros adotassem a padronização para publicação de estudos, como por exemplo o escore STARD (*Standards for Reporting of Diagnostic Accuracy* - www.consort-statement.org/stardstatement.htm) [99], o que garantiria clareza, rigor metodológico e possibilidade de comparação de resultados. A heterogeneidade detectada entre os ensaios e a ausência de estudos representativos de todas as regiões endêmicas, considerando a variabilidade dos parasitos, dos hospedeiros e a diversidade ambiental, limita a extrapolação de conclusões para todas as regiões do mundo. Mais estudos avaliando o desempenho dos vários testes sorológicos deveriam ser conduzidos nos continentes africano, asiático e americano a fim de confirmar as informações já reunidas na Europa e os nossos próprios dados. Em especial, ensaios utilizando testes rápidos baseados na identificação de anticorpos anti antígeno rK39 deveriam ser realizados nas várias regiões endêmicas antes de sua utilização de forma rotineira no diagnóstico de LV em regiões de alta prevalência do HIV.

Sobre a avaliação de fatores prognósticos, o emprego de medidas de desfecho mais precisas, grupos de pacientes controlados e tempo de seguimento adequado, certamente garantiriam informação mais confiável em relação a algumas condições que não puderam ser avaliadas - como o impacto dos diversos esquemas terapêuticos, antirretrovirais e leishmanicidas, sobre a taxa de recorrência de LV. O uso de marcadores moleculares quantitativos deve melhorar a precisão das estimativas de progressão da doença. Por outro lado, essa técnica impõe um novo desafio para sua validação: por sua alta sensibilidade, alternativas aos exames

parasitológicos como padrão ouro, tal como emprego da análise por classes latentes, precisarão ser discutidas e comparadas.

Embora a anfotericina B figure como a melhor opção dentre os tratamentos estudados para LV entre infectados pelo HIV, a presença de diferença na eficácia entre suas várias formulações e a dose ideal, em cada região endêmica, são questões que ainda precisam ser esclarecidas por ensaios clínicos prospectivos e randomizados. O tratamento com miltefosina, bem como as várias possibilidades de terapia combinada precisarão ser avaliados entre coinfectados com *Leishmania*-HIV.

Por fim, por exigência da própria metodologia de revisão sistemática, a atualização periódica destas revisões torna-se a partir de agora um compromisso, a fim de se manter o alinhamento necessário com a evidência científica disponível.

6 ANEXOS

6.1 **ANEXO I. Ficha para coleta de dados na avaliação inicial e seguimento**

Identificação

número na pesquisa _____

Número de registro _____

Data de nascimento: ___/___/___

sexo ○ masculino ○ feminino idade: _____

residência ○ BH/RM ○ Interior MG ○ outra ○ ig

escolaridade ○ analfab ○ ≤1º grau 2º grau comp/incompleto ○ 3º grau ○ ig

○ M0 ○ M1(final tto) ○ M2 ○ M4 ○ M6 ○ M8 ○ M10 ○ M12 ○ MR

Comorbidades

HIV/AIDS ○ não ○ sim ○ ig

HCV crônico ○ não ○ sim ○ ig

HBV crônico ○ não ○ sim ○ ig

Diagnóstico prévio esquistossomose ○ não ○ sim ○ ig

Diagnóstico de Doença de Chagas ○ não ○ sim ○ ig

Etilismo presente ou passado ○ não ○ sim ○ ig

Drogadição presente ou passada ○ não ○ sim ○ ig _____

outra comorbidade ○ não ○ sim ○ ig _____

Infecção HIV

Data do diagnóstico HIV: ____/____/_____ (dd/mm/aaaa)

Categoria transmissão:( ) 0-homo 1-hetero 4-transfusão 5-vertical 6-IVD 8-IG

Uso de TARV antes do dx LV primário ○ não ○ sim ○ ig

Data início uso primeiro esquema TARV: ____/____/_____ (dd/mm/aaaa)

ùltimo esquema TARV _____

LV último episódio ou atual

Data do diagnóstico ____/____/____ (data do primeiro exame positivo)

trata-se de LV primária ○ não ○ sim ○ ig

Data LV primária (se não for o 1º epidodio) _____/_____/_____ (dd/mm/aaaa)

Número episódios anteriores _____

Passado de infecções oportunistas antes de LV primária ○ não ○ sim ○ ig ○NA

Sintomas

Febre relatada ou termometrada ○ não ○ sim ○ ig

Citopenia (<3500 leuc ou < 120.000 plaq ou Hg < 11) ○ não ○ sim ○ ig

Visceromegalia ao exame físico (baço palpado ou fígado > 2 cm) ○ não ○ sim ○ ig

Teve dx LV (clínica + sorol + ou parasitológico+) ○ não ○ sim ○ ig

Tempo em dias desde o primeiro sintoma até o dx ___ ____ _____

Morbidade e fatores de gravidade

Apresentou <500 neutrofilos entre inicio sintomas e fim tto LV ○ não ○ sim ○ ig

Uso ATB (tto infecção bacteriana) entre inicio sintomas e fim tto LV ○ não ○ sim ○ ig

Recebeu transfusão qquer momento entre inicio sintomas e fim tto LV ○ não ○ sim ○ ig

Dias de internação: __ __ __

Internação CTI ○ não ○ sim ○ ig ○ NA

hemodiálise: ○ não ○ sim ○ ig ○ NA

Ventilação mecânica ○ não ○ sim ○ ig ○ NA

Tratamento LV último episódio ou atual

Medicação1: ( ) 1- Glucantime® 2- Anfote desox 3 – Anfo lipo 4-outro 8-ig 9-NA

Data do início:___/___/___ Data de término: ___/___/___

Dose dia em mg __ __ __ __

Duração do tratamento: __ __ __ dias (dias em que a medicação foi efetivamente administrada)

Medicação2: ( )

Data do início:___/___/___ Data de término: ___/___/___

Dose dia em mg __ __ __ __

Duração do tratamento: __ __ __ dias (dias em que a medicação foi efetivamente administrada)

Medicação3 ( )

Data do início:___/___/___ Data de término: ___/___/___

Dose dia em mg __ __ __ __

Duração do tratamento: __ __ __ dias (dias em que a medicação foi efetivamente administrada)

Avaliação de resposta clínica ao tratamento LV último episódio ou atual ao fim do tto (último dia +/- 3 dias)

Desaparecimento da febre (> 48 horas) ○ não ○ sim ○ ig ○ NA

Redução da esplenomegalia > 2 cm em relação ao M0 ○ não ○ sim ○ ig ○ NA

Aumento maior ou igual a 2g% na Hg em relação ao M0 ○ não ○ sim ○ ig ○ NA

Aumento maior ou igual 50% leucócitos em relação ao M0 ○ não ○ sim ○ ig ○ NA

Aumento maior ou igual 50% plaquetas em relação ao M0 ○ não ○ sim ○ ig ○ NA

Óbito em até 30 dias do diagnóstico LV último episódio ou atual

○ não ○ sim ○ ig ○ NA

Data____/_____/____ atribuído a LV ou tto ○ não ○ sim ○ ig ○ NA

Profilaxia secundária após LV primária

○ não indicada ○ indicada ○ ig ○ NA (se HIV negativo)

Medicação1: ( ) 1- Glucantime® 2- Anfo desox 3–Anfo lipo 4-outra_____

AVALIAÇÃO LABORATORIAL NA LV ÚLTIMO EPISÓDIO OU ATUAL

Teste ráp rK39 ○ neg ○ positivo ○ ig ou indeterminado ○ não realizado

Sorologia IFAT ○ neg ○ positivo ○ ig ou indeterminado ○ não realizado título: _____

Sorologia DAT ○ neg ○ positivo ○ ig ou indeterminado ○ não realizado título: _____

P. direta MO ○ neg ○ positivo ○ ig ou indeterminado ○ não realizado

Mielocultura ○ neg ○ positivo ○ ig ou indeterminado ○ não realizado

qPCR sangue: _____

número na pesquisa ( )

Acompanhamento clínico - Anamnese e exame físico

	M0	M1	M2	M4	M6	M8	M10	M12
DATA	_/__/_	__/__/	_/__/_	_/__/_	_/__/_	_/__/_	_/__/_	_/__/_
Febre								

Peso								
Fígado (cm)								
Baço (cm)								
0-sem doença 1-recidiva 2-abandono/falta 3-óbito								

	M0	M1	M2	M4	M6	M8	M10	M12
TARV (> 4 sem)								
adesão TARV								
em profilaxia usou alguma vez no período		**X**						
medicação profilática*		**X**						
Prof regular se intervalo max 4 sem		**X**						
intervalo médio da profilaxia média dos intervalos do período		**X**						

***** 1- Glucantime® 2- Anfote desox 3 – Anfo lipo 4-outro 8-ig 9-NA

Exames laboratoriais

	M0	M1	M2	M4	M6	M8	M10	M12
Exames *	_/__/_	__/__/	_/__/_	_/__/_	_/__/_	_/__/_	_/__/_	_/__/_
Hb								
Ht								
Plaquetas								
Leucócitos								
neut/linf/eos								

Exames §	_/__/_	__/__/	_/__/_	_/__/__	_/__/_	_/__/_	_/__/_	_/__/_
Cd4								
Exames §	_/__/_	__/__/	_/__/_	_/__/_	_/__/_	_/__/_	_/__/_	_/__/_
PCR								
Log								

* considerar os exames realizados na data ou em ± 15 dias da data da avaliação programada, o mais próximo
§ considerar os exames realizados na data ou em -90 a +30 dias da data da avaliação programada, o mais próximo

6.2 ANEXOII Ficha de coleta de dados morbimortalidade dos casos LV

Identificação

Registro _____

Peso _____ altura_____ IMC_____

Morbidades

(presença em relato à admissão ou ocorrência em qualquer momento até 5º dia de tratamento)

Presença de sangramento ( ) 0 não 1sim 8-ignorado

Tipo de sangramento: ( )

1-pele (petequia/equimose) 2- mucosa (gengivorragia/epistaxe) 3- TGI (melena/enterorragia/hematemese) 4- hematuria 5- cavitário (derrame) 8-ignorado

presença de edema periferico ( ) 0-não 1- sim 8-ignorado

vômitos ( ) 0-não 1- sim 8-ignorado

diarreia ( ) 0-não 1- sim 8-ignorado

ictericia observada ao exame clínico ( ) 0-não 1- sim 8-ignorado

dispneia ( ) 0-não 1- sim 8-ignorado

choque/hipotensão ( ) 0-não 1- sim 8-ignorado

registro de desnutrição ( ) 0-não 1- sim 8-ignorado

Exames

BT (bilirrrubina total) ___ ____ ____

BD (bilirrubina direta) ___ ____ ____

BI (bilirrubina indireta) ___ ____ ____

TGO ___ ____ ____

TGP ___ ____ ____

Creatinina ___ ____ ____

AP ___ ____ ____

RNI___ ____ ____

Albumina ___ ____ ____

Toxicidade ao tratamento

Medicação1: ( ) 1- Glucantime® 2- Anfote desox 3 – Anfo lipo 4-outro 8-ig 9-NA

Data do início:___/___/___ Data de término: ___/___/___

Dose dia em mg __ __ __ __ Duração do tratamento: __ __ __ dias

Apresentou alguma toxicidade ao tratamento ○ não ○ sim ○ ig ○ NA

Qual toxicidade ( )

1- renal 2- hepática 3- cardíaca 4- pancreática 5- músculo-articular 6- hipocalemia 7-outra/associação _____8-ig 9-NA

Medicação2: ( ) 1- Glucantime® 2- Anfote desox 3 – Anfo lipo 4-outro 8-ig 9-NA

Data do início:___/___/___

Dose dia em mg __ __ __ __

Duração do tratamento: __ __ __ dias

Data de término: ___/___/___

Apresentou alguma toxicidade ao tratamento ○ não ○ sim ○ ig ○ NA

Qual toxicidade ( ) 1- renal 2- hepática 3- cardíaca 4- pancreática 5- músculo-articular 6- hipocalemia 7-outra/associação _____8-ig 9-NA

Medicação3: ( ) 1- Glucantime® 2- Anfote desox 3 – Anfo lipo 4-outro 8-ig 9-NA

Data do início:___/___/___

Dose dia em mg __ __ __ __

Duração do tratamento: __ __ __ dias

Data de término: ___/___/___

Apresentou alguma toxicidade ao tratamento ○ não ○ sim ○ ig ○ NA

Qual toxicidade ( ) 1- renal 2- hepática 3- cardíaca 4- pancreática 5- músculo-articular 6- hipocalemia 7-outra/associação _____8-ig 9-NA

Definição de toxicidade (considerar o parâmetro desde que não presente na avaliação pré tto)

1- Renal: elevação de 50% ou mais da creatinina em relação ao pré tto ou creat > 2mg%
2- Cardíaca: ICC (diagnóstico clínico ou a o ECO), relato de alguma arritmia, incluindo prolongamento de QTc acima do normal (homem> 0,45 mulher>0,47)
3- Pancreática: ao menos uma das duas enzimas (lípase e amilase) > 6 vezes o LSN ou diagnóstico clínico + qquer elevação enzimas
4- Músculo-articular: relato de mialgia ou artralgia/artrite ou el evação CK > 6 vezes LSN
5- Hipocalemia: K < 3,5 em qualquer momento após início da anfo, até 5 dias do término

6.3 **ANEXO III. Ficha de coleta de dados dos casos controles**

Identificação

número na pesquisa _____

Definição diagnóstica

Teve uma definição diagnóstica para explicar febre e/ou citopenia e/ou visceromegalia : ○ não ○ sim ○ ig ○ NA

Dias entre inicio dos sintomas e definição diagnóstica ____ _____ _____

O diagnóstico foi estabelecido com base em ( )

1- exame microbiológico ou histopatológico

2- exame de imagem

3- exame sorológico ou outro exame de patologia clínica

4- quadro clínico compatível + afastadas outras hipóteses

5- quadro clínico compatível + afastadas outras hipóteses + prova terapêutica positiva

6- mais de um dos anteriores _____

8-ignorado

O paciente recebeu terapia ativa para leishmania durante o período de investigação

○ não ○ sim ○ ig

Medicação ( ) 1- Glucantime® 2- Anfote desox 3 – Anfo lipo 4-outro 8-ig 9-NA

Data do início:___/___/___ Data de término: ___/___/___

Dose dia em mg __ __ __ __

Duração do tratamento: __ __ __ dias (dias de medicação efetivamente administrada)

O paciente recebeu alguma terapia específica para a condição provável causa dos sintomas ○ não ○ sim ○ ig

Avaliação médica entre 30 +/- 15 dias após inicio da investigação para LV

Existe avaliação médica em 30 ou mais dias após inicio da investigação para LV?

○ não ○ sim ○ ig

Desaparecimento da febre (> 48 horas) ○ não ○ sim ○ ig ○ NA

Redução da esplenomegalia > 2 cm em relação ao M0 ○ não ○ sim ○ ig ○ NA

Aumento maior ou igual a 2g% na Hg em relação ao M0 ○ não ○ sim ○ ig ○ NA

Aumento maior ou igual 50% leucócitos em relação ao M0 ○ não ○ sim ○ ig ○ NA

Aumento maior ou igual 50% plaquetas em relação ao M0 ○ não ○ sim ○ ig ○ NA

Óbito em até 30 dias após o início da avaliação LV ○ não ○ sim ○ ig ○ NA

Data____/_____/____

Causa do óbito: _____

Diagnóstico definido ( )

1- HIV
2-micobacteria _____

3-doença fungica _____

4-doença infecciosa outra alem das especificadas_____

5- doença clínica não infecciosa _____

6- esquistossomose (FHE) _____

7- hepatopatica cronica/hiperesplenismo não FHE_____

8- ignorado

10- duas ou mais das anteriores

6.4 **ANEXO IV. TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Eu, _____,

fui convidado a participar da pesquisa "Intensidade de infecção, persistência parasitária e perfil imune como preditores de evolução clínica de leishmaniose visceral entre pacientes infectados ou não pelo HIV".

Leishmaniose visceral ou Calazar é uma doença causada por um parasito chamado Leishmania, que tem tratamento mas pode apresentar recaída, principalmente nos 12 meses seguintes ao tratamento.

A pesquisa tem como objetivo estudar a quantidade de parasito (chamada de parasitismo) no sangue e na medula óssea e a resposta do corpo contra esta infecção (chamada de resposta imune), antes e após o tratamento contra a leishmaniose e assim avaliar o risco de reaparecimento da doença. Será avaliada a utilidade de um novo exame para a detecção da infecção, chamado Reação em Cadeia da Polimerase em tempo real (PCR). Este exame pode identificar o parasito de forma mais sensível, antes mesmo da doença se manifestar e pode ser realizado no sangue ou no material colhido da medula do osso. Assim, nossa intenção é melhorar o entendimento das recaídas da doença, para conseguir cuidar de maneira mais adequada dos outros doentes que venham a ter leishmaniose visceral no futuro.

Para participar da pesquisa, você precisará ser avaliado por um médico da nossa equipe no início do acompanhamento e depois a cada 60 dias nos próximos 12 meses. Em cada avaliação, você deverá conversar com um médico da nossa equipe e ser examinado. Será também realizada uma coleta de sangue (cerca de 5 ml) para ser analisado no Laboratório de Pesquisas Clínicas do Centro de Pesquisas René Rachou. No caso de realização do exame de punção de medula óssea antes do início do tratamento, uma pequena quantidade do material coletado também será reservada para a realização do exame de PCR. Se você apresentar sinais de recaída da doença, tais como febre, aumento do volume abdominal e perda de peso, você deve procurar este hospital e, neste caso, depois de examinado, serão realizados coleta de sangue e nova amostra de medula óssea para exames.

A técnica para coletar o aspirado de medula óssea consiste em uma punção por agulha própria na crista ilíaca (osso da bacia), realizada com anestesia local. A coleta de sangue (punção venosa) terá a mesma técnica utilizada para colher os exames comuns. Os riscos da realização destes procedimentos são pequenos. Você poderá sentir dor leve momentânea no local da punção e depois poderá formar um pequeno hematoma (mancha roxa). Caso apresente qualquer problema, você será avaliado por um médico da pesquisa e receberá todo o atendimento necessário.

Em qualquer momento, você poderá decidir sair da pesquisa. Caso saia, não haverá nenhuma diferença no tratamento que você está recebendo no Hospital Eduardo de Menezes. Você não receberá nenhuma gratificação para participar da pesquisa. As informações obtidas através dessa pesquisa serão confidenciais. Somente você e nós, do grupo da pesquisa, teremos acesso às informações. Os dados não serão divulgados de forma a identificá-lo.

Você receberá uma cópia deste termo, onde consta o telefone do pesquisador principal e do Comitê de Ética em Pesquisa do Centro de Pesquisa René Rachou e do Hospital Eduardo de Menezes. Você pode esclarecer suas dúvidas sobre o projeto e sua participação, agora ou a qualquer momento.

Declaro que entendi os objetivos, riscos e benefícios da minha participação e concordo em participar

_____ _____

Assinatura do participante Assinatura do pesquisador

Belo Horizonte, _____ de _____ de _____.

Pesquisadores:

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