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CARACTERIZACIÓN PARASITOLÓGICA E INMUNOLÓGICA DE LA LEISHMANIASIS VISCERAL EN EL ESTADO DE AMHARA, ETIOPÍA

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

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Bajo la dirección de la doctora

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Caracterización parasitológica e inmunológica de la leishmaniasis visceral en el Estado de Amhara, Etiopía.

Tesis doctoral

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RESUMEN

La leishmaniasis visceral (LV) o kala-azar es una enfermedad infecciosa que resulta fatal sin diagnóstico y tratamiento adecuado. Está causada por parásitos pertenecientes al complejo *Leishmania donovani* y es transmitida por dípteros hematófagos, concretamente hembras de diversas especies del género *Phlebotomus* en el Viejo Mundo y del género *Lutzomyia* en el Nuevo Mondo. Se estima que la incidencia mundial de LV es de 500 000 casos, de los cuales el 90% ocurre en diferentes países que se encuentran en tres grandes focos: el subcontinente indio (Bangladesh, India y Nepal), y el cuerno de África (Sudan y Etiopía), donde se considera que la transmisión es principalmente antroponótica, y América (Brasil), donde se considera una zoonosis, con el perro como principal reservorio. Cada año mueren entre 50 000 y 60 000 personas debido a la LV, cuya tasa de mortalidad, entre las enfermedades parasitarias, sólo es superada por la malaria. En los últimos años, se ha documentado la (re)emergencia de la LV en numerosos países. El número de casos en las zonas endémicas ha aumentado y además están apareciendo nuevos focos en regiones donde previamente no se había detectado la enfermedad. Entre los factores responsables destacan: los cambios medioambientales, la inmunodepresión, el movimiento en masa de población no inmune a áreas endémicas y la resistencia a los antimoniales pentavalentes.

En Etiopía se calcula que la incidencia de LV es 4 500 a 5 000 casos. Este país ostenta el mayor porcentaje de coinfección *Leishmania*/VIH; entre los años 1998-2007 la proporción de casos de LV asociados a infección por VIH aumentó del 18,5 al al 41%. Recientemente, el norte del país ha sufrido un brote de LV que costó la vida de centenares de personas. El 90% de los afectados tenía entre 8 y 45 años de edad y más del 20% sufrían malnutrición. Estos hechos demuestran que la LV está convirtiéndose en una preocupación creciente para la salud pública de Etiopía, siendo prioritario un mayor conocimiento de su epidemiología en este país para poder diseñar un programa de vigilancia y control adecuado.

En ausencia de inmunodepresión, sólo un pequeño porcentaje de los infectados desarrolla la enfermedad, mientras que la mayoría permanecen asintomáticos. Dado que los portadores asintomáticos podrían actuar como reservorios, el conocer la prevalencia de infección en este grupo contribuiría a una mejor comprensión de la dinámica de la transmisión, y por tanto sería de gran utilidad a la hora de desarrollar estrategias de control.

Actualmente se considera necesario el diseño de un programa de control eficaz de la LV en Etiopía, que utilice herramientas que permitan determinar adecuadamente la exposición al parasito (para poder estimar la prevalencia e incidencia), y que identifique factores de riesgo asociados a infección y enfermedad. En este último caso merecen especial atención aquellos que afectan a la respuesta inmune.

Para contribuir a esta iniciativa UBS-Optimus Foundation financió el proyecto *Visceral leishmaniasis and malnutrition in Amhara State, Ethiopia*, que contempla la caracterización nutricional, inmunológica y parasitaria de la población infantil del Estado de Amhara, área en la que se registró el brote de LV arriba mencionado. Y es dentro del marco de este proyecto en el que se plantea el presente trabajo, cuyos objetivos, asociados a distintos artículos científicos, se detallan a continuación:

1- Evaluación de herramientas diagnósticas para la detección de la infección asintomática por *Leishmania* (Artículo 1).

2- Determinar la prevalencia post brote de LV activa e infección asintomática para establecer si aún existe transmisión activa (Artículo 2).

3- Identificar factores de riesgo asociados a la infección (Artículo 3).

4- Estudiar la influencia de la malnutrición en la inmunidad adaptiva y su posible asociación a la susceptibilidad a infección y/o enfermedad (Artículo 4).

La población de estudio de este trabajo ha estado formada por los niños de 4 a 15 años de edad de los distritos de Libo Kemkem y Fogera (Estado de Amhara, Etiopía). Los datos presentados se han obtenido a través de un estudio por conglomerados multi-etapas y del registro del centro de tratamiento de LV de Addis Zemen (Estado de Amhara). Los datos sociodemográficos, antropométricos, clínicos y las muestras biológicas utilizadas en el estudio fueron obtenidos por profesionales de la salud debidamente entrenados para realizar este.

La población de estudio utilizada para evaluar la utilidad de las herramientas diagnósticas en la detección de infección asintomática, se seleccionó de sub-distritos con una alta prevalencia (≥ 8 casos) después del brote de LV ocurrido en 2004-2005, según el registro del centro de tratamiento de LV de Addis Zemen (datos de 2008) (Población 1). Se evaluaron dos aproximaciones serológicas (test de aglutinación directa-DAT, y tira inmuno-cromatográfica rápida basada en el antígeno recombinante rK39-rK39-ICT) y una determinación de la respuesta de hipersensibilidad retardada

(test de la leishmanina-LST). Para ello se encuestaron 605 niños sin LV previa y se detectó infección asintomática en 61 de ellos (10.1%). Se encontraron, además, durante este estudio, tres casos activos de LV. Por tanto se pudo confirmar que existe transmisión activa de *Leishmania* en los sub-distritos estudiados. Además se comprobó que el uso combinado de DAT y LST es la mejor opción para detectar la infección asintomática.

Para determinar la prevalencia en el área de estudio se muestrearon todos los sub-distritos en los que había habido, de acuerdo al registro del centro de tratamiento de LV de Addis Zemen, al menos un caso de LV durante el brote (Población 2). De un total de 386 niños encuestados, sólo se encontró un caso activo de LV. Mientras que la prevalencia de infección asintomática fue del 1.02%. Se observó que la prevalencia aumentaba con la edad y que, además, era mayor entre el sexo masculino. La baja prevalencia observada (tanto de casos activos como de infección asintomática) indica que las condiciones que originaron el brote ya no persisten, y que actualmente se da una situación de baja transmisión. No obstante, la ausencia de datos sobre las causas que originaron el brote hace necesario mantener un sistema de vigilancia para poder predecir un nuevo brote o incremento de los casos.

La identificación de factores de riesgo asociados con la infección se realizó estudiando la Población 1. Se analizaron los datos sociodemográficos y nutricionales de la población infantil estudiada con respecto a la infección asintomática por *Leishmania*. Se realizaron análisis multi-variante y uni-variante. Los factores mostraron una asociación positiva con la infección: mayor edad, sexo masculino, malnutrición aguda, el hábito de dormir fuera de la casa, el cuidado de ganado, número creciente de miembros en la familia, historia previa de LV en algún miembro de la familia, casa con techo de paja y casa con grietas en las paredes. Como factores protectores se encontraron: posesión de un mayor número de cabezas de ganado y gallinas por familia. En conclusión, estos resultados indican que factores modificables como las condiciones de la vivienda, la malnutrición y los hábitos personales influyen en la transmisión de LV, subrayando la necesidad de educación y movilización social para mitigar el problema. Por otra parte, la protección asociada al aumento del número de cabezas de ganado que posee una familia podría estar indicando bien un mayor grado de riqueza (con una influencia positiva en la salud y otros elementos físicos del hogar), o un comportamiento zoofílico del vector, de manera que el ganado hiciese de barrera a la transmisión; no obstante cualquiera de estas explicaciones neceistaría de un estudio más detallado.

Para estudiar el impacto de la malnutrición sobre la inmunidad se realizó un análisis de parámetros hematológicos, poblaciones linfocitarias de sangre periférica, niveles séricos de PGE2 y de citoquinas, así como los niveles de estas últimas en el sobrenadante de cultivos de células mononucleadas de sangre periférica (PBMC) estimuladas con fitohemaglutinina (PHA) en niños de la población de estudio (Población X). Se observó que la malnutrición severa en el sexo masculino se asocia a un valor de hematocrito (HCT), niveles de hemoglobina (HGB) y leucocitos circulantes (WBC) más bajos que en los no malnutridos. Sin embargo, no se observaron diferencias para estos parámetros dentro del sexo femenino. Independientemente del sexo, los individuos con malnutrición severa mostraron una disminución en el número absoluto de leucocitos circulantes y de las sub-poblaciones de linfocitos T (CD4+ y CD8+) y B en comparación con los individuos no malnutridos. El análisis de sobrenadantes de cultivo de PBMC estimuladas con PHA mostró que los no malnutridos tenían una mayor concentración de IL-10, IL-2 e IFN-γ en comparación con los que presentaban malnutrición severa. Por el contrario, los sueros de individuos no malnutridos presentaron títulos más bajos de PGE2 en comparación con los malnutridos. Al integrar el factor infección (determinado por DAT) se observó que la capacidad de las PBMC de producir IL-10 e IFN-γ de manera constitutiva era mayor en los no malnutridos que en los malnutridos, independientemente del estatus de infección. Lo que indica una mejor condición inmunológica en los individuos no malnutridos.

INTRODUCCIÓN

La leishmaniasis representa un complejo de enfermedades con una importante diversidad clínica y epidemiológica. Está causada por diversas especies de protozoos del género *Leishmania* que son transmitidos a través de la picadura de hembras de flebótomo, pertenecientes al género *Phlebotomus* en el Viejo Mundo y al género *Lutzomyia* en el Nuevo Mundo. Las diferentes formas clínicas dependen del resultado de la interacción entre la especie infectante y la respuesta inmune del huésped; y varían desde distintas manifestaciones tegumentarias (leishmaniasis cutánea, cutáneo-difusa o mucosa) a la leishmaniasis visceral, una afección sistémica que es mortal sin tratamiento. La leishmaniasis se encuentra en el grupo de las *Enfermedades Olvidadas*, debido a los limitados recursos invertidos en su diagnóstico, tratamiento y control, y a su fuerte relación con la pobreza. (WHO, 2010; Bern *et al.,* 2008).

I- EPIDEMIOLOGÍA DE LA LEISHMANIASIS

La epidemiología de la leishmaniasis depende de la especie de *Leishmania*, las características ecológicas de las áreas de transmisión, incluyendo la biología del vector, y la exposición actual y pasada de la población humana al parásito. La leishmaniasis es endémica en 98 países o territorios, con más de 350 millones de personas en riesgo, y una prevalencia (probablemente muy subestimada) de 12 millones. De acuerdo a los datos publicados la incidencia se estima en 2 millones de casos nuevos al año (0.5 millones debidos a la leishmaniasis visceral, y 1.5 millones debidos a las diferentes formas tegumentarias). Genera una pérdida de 2 357 000 años de vida ajustados por discapacidad, lo que sitúa a la leishmaniasis cuarta en cuanto a morbilidad dentro de las enfermedades tropicales y novena en un análisis global de las enfermedades infecciosas. La leishmaniasis visceral (LV) causa unas 50 000 muertes al año, una tasa superada, entre las enfermedades parasitarias, sólo por la malaria (Desjeux, 2004; WHO, 2010).

La leishmaniasis presenta una amplia distribución, existiendo transmisión a humanos en 5 continentes. No obstante, la mayor carga de enfermedad se localiza en unos focos concretos. El 90% de los casos de LV ocurre en Bangladesh, Brasil, Etiopía, India, Nepal y Sudán; mientras que en Afganistán, Argelia, Arabia Saudí, Brasil, Irán, Perú y Sudán se da el 90% de los casos de leishmaniasis tegumentaria. Por otra parte, la distribución es dinámica, de manera que varias áreas endémicas muestran una amplia fluctuación en la incidencia a lo largo del tiempo, lo que en

ocasiones se atribuye a eventos específicos como el desplazamiento de poblaciones y factores climáticos. Cambios ambientales, climáticos y socioeconómicos podrían expandir el rango geográfico de la transmisión de la leishmaniasis (Alvar, *et al*., 2006; Chappuis *et al*., 2007).

Un fenómeno de creciente preocupación es la co-infección *Leishmania*/VIH, que intensifica la carga de enfermedad, causando formas más severas y más difíciles de manejar. Si bien en Europa la incidencia de esta co-infección disminuyó desde finales de los 90, en otras partes del mundo está aumentando lentamente, según la pandemia de VIH se expande a las áreas rurales endémicas de leishmaniasis. Particularmente, en el norte de Etiopía la tasa de VIH en enfermos de leishmaniasis visceral incrementó desde el 19% en los años 1998-9 al 34% en 2006-7. En Brasil, India, Nepal y Sudán la prevalencia aún se mantiene por debajo del 10%, pero se espera que aumente (WHO, 2010).

I.1- Ciclo biológico

La leishmaniasis está presente en ecosistemas extremadamente diversos, con adaptaciones específicas para cada especie de vector. Además, *Leishmania* es capaz de infectar una amplia variedad de mamíferos. Desde el punto de vista de la fuente de infección en humanos, la leishmaniasis puede clasificarse en dos amplias categorías: i) leishmaniasis zoonótica, en la que el reservorio de infección lo constituyen animales salvajes o domésticos; y ii) leishmaniasis antroponótica, en la que el reservorio es el hombre. Así, la existencia de la leishmaniasis queda determinada por una serie de condiciones epidemiológicas que permiten el contacto del parásito con el vector y con los huéspedes vertebrados, llevándose a cabo el desarrollo completo y continuo del ciclo biológico de *Leishmania* (Figura 1) (Marzinovsky y Schurenkowa, 1924; Adler, 1940).

Leishmania (Leishman, 1903; Donovan, 1903; Wright, 1903) es un parásito digénico y dimórfico, que realiza parte de de su ciclo vital en el tubo digestivo del flebótomo, donde se encuentra en la forma promastigote (1.5-3 μm x 10-20 μm), presentando un flagelo anterior, y en el huésped vertebrado, parasitando las células fagocíticas del sistema retículo-endotelial en la forma amastigote (2.5 x 6.8 μm), con un flagelo residual. El modo en que se multiplican ambas formas del parásito es por fisión binaria (Bryceson, 1996).

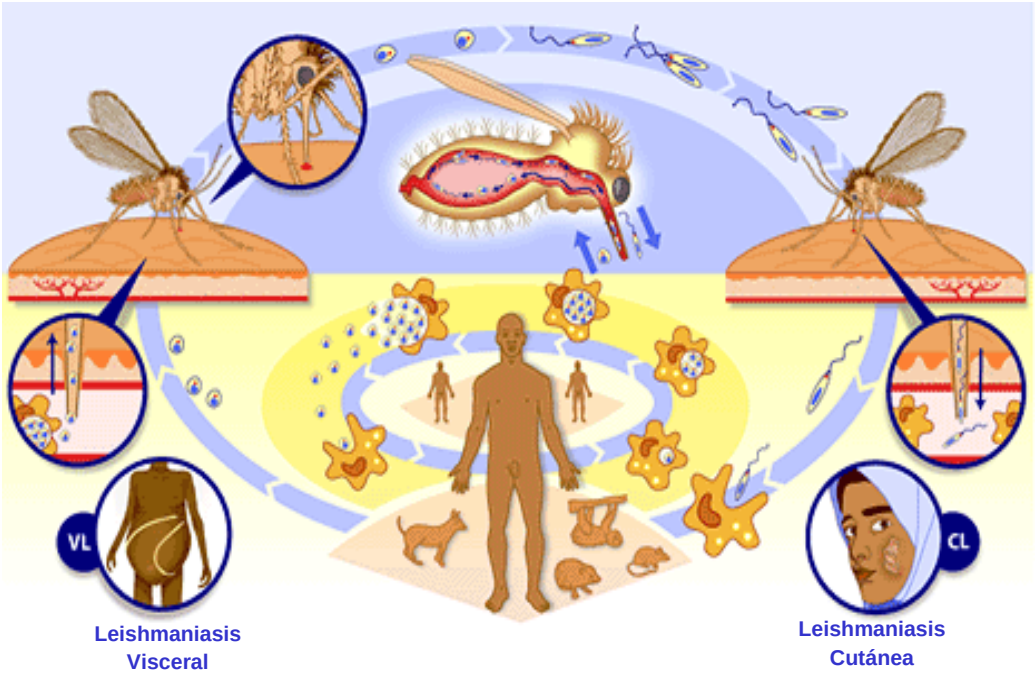

Figura 1. Ciclo biológico de *Leishmania* (www.who.int/tdr/diseases/leish/default.htm)

Leishmania es transmitida al huésped mamífero mediante la picadura de una hembra de flebótomo infectada. Las hembras de flebótomos vectores necesitan ingerir sangre para que se produzca el desarrollo completo de sus huevos. La preferencia de los vectores por los diferentes vertebrados varía de acuerdo a la especie y a la disponibilidad de huéspedes. Durante el proceso de alimentación sobre el huésped, el flebótomo introduce, además de los parásitos, su saliva. Esta, junto con los proteofosfoglicanos del parásito se cree que ejercen un papel importante en el establecimiento del parásito en la piel (WHO, 2010). Cuando la hembra del flebótomo toma sangre de un mamífero infectado, que presenta parasitemia, en el caso de la leishmaniasis visceral, o de la propia úlcera en las cutáneas, esta puede ingerir macrófagos infectados con amastigotes de *Leishmania*. En el interior del aparato digestivo del vector, los amastigotes se transforman en promastigotes; estos se multiplican por fisión binaria y van migrando hacia la porción anterior del aparato digestivo, donde se transformarán en promastigotes metacíclicos, que es la forma infectiva que transmitirá el vector (Molyneux y Killick-Kendrick, 1987).

Al inocular los parásitos en la dermis del huésped, aquellos que sobreviven a la acción del complemento se adhieren rápidamente a las células residentes o a las

reclutadas pertenecientes al linaje de monocitos/macrófagos (Moll *et al*., 1995). La adhesión está mediada por receptores de membrana en las células diana que se unen a factores del complemento adheridos al parásito; posteriormente este penetra al interior celular mediante fagocitosis. *Leishmania* queda entonces englobada en un fagolisosoma que evoluciona a lo largo de la ruta fagocítica hasta convertirse en una vacuola parasitófora, con unas características finales entre endosoma tardío y lisosoma (Amer y Swanson, 2002; Waller y McConville, 2002). Una vez en la vacuola, y tras 2 a 5 días, los promastigotes se transforman en amastigotes (Antoine *et al*., 1998; *Handman*, 1999). Los amastigotes se dividen en el interior de la vacuola parasitófora hasta que la célula no puede acumular más y se rompe liberándolos al exterior, donde son captados por otras células competentes.

En el caso de las especies o cepas viscerotrópicas, una vez la infección se ha establecido, los parásitos pueden persistir en el huésped durante toda su vida en las células del sistema retículo-endotelial, en médula ósea o bazo, bien en forma activa, causando enfermedad, o acantonados cursando una infección subclínica, asintomática, con la posibilidad de reactivarse y causar patología si consiguen escapar al control del sistema inmune del huésped. Sin embargo, las especies o cepas dermotrópicas generalmente se mantienen localizadas junto al punto de inoculación, causando la enfermedad cutánea. Cualquier dispersión de las especies dermotrópicas suele ser tardía y sólo hacia porciones de piel adyacentes (produciendo lesiones satélites), o hacia el tejido o nódulos linfáticos próximos. Algunas especies del subgénero *Viannia* (como *L. braziliensis*) migran a la mucosa oro-faríngea donde pueden permanecer en estado latente durante años hasta reactivarse y causar lesiones mucosas destructivas (Alvar, 2001).

II- LEISHMANIASIS VISCERAL

La Leishmaniasis Visceral (LV), también llamada Kala-azar (KA), es endémica en 65 países. Actualmente se reconocen dos especies de *Leishmania* asociadas a esta enfermedad: *Leishmania donovani* y *Leishmania infantum*. La primera se asocia a una transmisión antroponótica y se distribuye en los principales focos del sub-continente indio y el este de África, si bien hay datos que indican que en determinadas áreas de este último foco la transmisión podría ser antropozoonótica. *L. infantum* se asocia a una transmisión zoonótica, con el perro como principal reservorio, y se distribuye principalmente por toda la cuenca mediterránea, algunas regiones de Oriente Medio y Asia, y por Sudamérica (WHO, 2010) (Figura 2).

Figura 2. Distribución geográfica de la LV (WHO, 2010).

II.1- (Re-)emergencia de la leishmaniasis visceral

La LV es una enfermedad dinámica y las circunstancias asociadas a su transmisión varían de un modo continuo en función de los cambios asociados al ambiente, y del comportamiento humano. De acuerdo con la OMS los programas de control de la leishmaniasis activos son escasos y la mortalidad y morbilidad asociadas a la leishmaniasis muestran una preocupante tendencia al alza en todo el Mundo (WHO, 2010).

En los últimos años, se ha documentado la emergencia de la LV en diversas áreas en las que no se había descrito antes la presencia de la enfermedad, como en la ciudad de Posadas, Argentina (Salomón *et al*., 2008), el estado de Amhara, noroeste de Etiopía (Alvar *et al*., 2007), o Bután (Bhattacharya *et al*., 2010). Así como la re-emergencia en focos que se consideraban controlados en Uzbekistán y Tajikistán (Alam *et al*., 2009). Y la rampante propagación de la LV urbana en Brasil (Maia-Elkhoury *et al*., 2008).

Entre los factores de riesgo asociados a la re-emergencia de la LV se encuentran aquellos relacionados con el ambiente, que aumentan la exposición al vector y/o estrechan el contacto con los reservorios, pero también están aquellos que,

en el huésped, facilitan la evolución de la infección a la enfermedad, y la propagación en la comunidad.

II.1.1- Factores ambientales

La leishmaniasis se caracteriza por una distribución en microfocos, este tipo de distribución se debe a condiciones micro-ecológicas que afectan al vector, el parásito y al reservorio. En función de la eco-epidemiología de cada foco en particular. Las alteraciones del ambiente, de origen natural o por acción humana, pueden generar un aumento o disminución de la incidencia de la enfermedad. Entre los cambios ambientales que afectan la incidencia se encuentran la urbanización, la domesticación de los ciclos de transmisión y la incursión de asentamientos humanos en entornos forestales (WHO, 2010).

II.1.2- Cambio climático

La distribución de la leishmaniasis presenta una asociación muy fuerte con el clima, estando muy afectada por factores como la lluvia, la temperatura atmosférica y la humedad. Estos factores tienen efecto en la distribución, supervivencia y tamaño de población de vectores y reservorios. Además, grandes efectos climáticos, como las sequías o inundaciones pueden provocar movimientos masivos de población hacia áreas endémicas, incrementando el número de individuos susceptibles (WHO, 2010).

II.1.3- Movimientos de población

Los movimientos de población, por causas ambientales o provocadas por la acción del hombre, están detrás del origen de algunas epidemias de LV; bien al poner a una población en contacto con un ciclo de transmisión, o al introducir un número importante de personas infectadas en un área no endémica en la que existe la posibilidad de transmisión vectorial. Un ejemplo dramático fue la epidemia de LV en la provincia de *Western Upper Nile,* en el sur de Sudán, asociada a la movilización de población a un área de transmisión zoonótica, provocada por la guerra civil que sufrió el país (1984-1994), y generando una epidemia que causó la muerte de 100 000 personas (WHO, 2010; Zijlstra y El-Hassan, 2001).

II.1.4- Inmunodepresión

La respuesta inmune innata juega un papel crucial en la resistencia del huésped a la infección por *Leishmania*. Esta respuesta actuaría tanto en el control de la multiplicación del parásito en la fase inicial de la infección, como en la generación de una cascada inmunoreguladora dirigida a ejercer una respuesta específica contra el parásito (Peruhype-Magalhães *et al*., 2005). En el caso de la LV, hay dos fenómenos que, al alterar esta respuesta inmune, están especialmente relacionados con un mayor riesgo de desarrollo de la enfermedad: la co-infección con VIH y la malnutrición.

El desarrollo de la pandemia de VIH/SIDA durante las últimas décadas ha modificado el espectro de la LV, tanto a nivel clínico como epidemiológico. Este fenómeno surge como consecuencia del solapamiento de ambas infecciones, que adquiere mayor importancia en los últimos años como consecuencia de la ruralización del SIDA y la urbanización de la leishmaniasis. En los pacientes co-infectados los dos patógenos ejercen un efecto sinérgico, lo que tiene implicaciones muy importantes en su expresión y propagación. El tiempo de desarrollo de SIDA se ve acelerado y la leishmaniasis puede no tener una presentación clásica. Estos pacientes presentarán una pobre respuesta a la terapia y una elevada tasa de recaídas; así como una elevada parasitemia y una serie de manifestaciones atípicas que dificultarán y retrasarán el diagnóstico. Así, los enfermos co-infectados engrosarían el número de reservorios humanos en áreas donde la transmisión es antroponótica. Pero además, las mismas características podrían ayudar a crear nuevos focos de transmisión antroponótica en áreas donde la LV es zoonótica. Quizás un ejemplo de la magnitud de este problema lo constituye el sur de Europa, que fue el paradigma de la co-infección *Leishmania*/VIH, encabezando la lista de casos reportados, durante la primera década de la pandemia de VIH/SIDA, y donde se observó que la prevalencia de LV en enfermos de SIDA era entre 100 y 2000 veces mayor que en individuos inmunocompetentes u otros grupos de inmunodeprimidos no VIH+ (Desjeux y Alvar, 2003; Molina *et al*., 2003; Cruz *et al*., 2006-a; Alvar *et al*., 2008).

Existen fuertes evidencias que indican un vínculo entre la malnutrición y un déficit de las respuestas immunes innata y adaptativa, de manera que en casos de deficiencia nutricional la LV presentaría formas más severas y letales. Particularmente en niños menores de 5 años la malnutrición juega un papel significativo en la evolución clínica de la LV. Si bien las bases que explican la asociación entre malnutrición y LV no están claras, se sabe que la malnutrición genera inmunodepresión y que esta es un factor de riesgo para el desarrollo de la enfermedad. La lactancia materna se asocia

con una mayor posibilidad de permanecer asintomático tras la infección, mientras que un peso bajo al nacer se asocia con una mayor posibilidad de desarrollar la enfermedad tras la infección. Del mismo modo, los niños que presentan bajas medidas antropométricas tienen mayor riesgo de desarrollar LV que los sanos (Maciel *et al*., 2008; Malafaia, 2009). A nivel epidemiológico se ha descrito el papel de la malnutrición en el desarrollo de epidemias de LV en el este de África; un claro ejemplo es el descrito por Marlet *et al*. (2003), que describen un brote de LV que afectó a 904 individuos entre mayo del año 2000 y agosto del 2001 en los distritos de Wajir y Mandera en una región fronteriza entre Kenia, Somalia y Etiopía.

II.1.5- Fallo terapéutico y resistencia a fármacos

La quimioterapia es crítica tanto para el paciente como para reducir el número de reservorios en áreas donde la transmisión es antroponótica. De manera que la monitorización del acceso al tratamiento en las diversas áreas endémicas es una pieza clave a la hora de desarrollar un programa de control, esta estrategia debe incluir también el control de la calidad del fármaco. Un acceso no controlado al mismo puede llevar a un uso incorrecto, tratamiento con dosis sub-óptimas, fallo terapéutico y, a largo plazo, el desarrollo de resistencias. Un fenómeno que ha merecido especial atención ha sido el desarrollo de resistencia al tratamiento con antimoniales pentavalentes (Sb^V). Estos han sido la primera opción para tratar la LV durante las últimas siete décadas, sin embargo en determinadas áreas de India y Nepal, el fallo terapéutico asociado a los Sb^V llega al 60%; lo que implica un mayor número de individuos infectados y capaces de actuar como reservorios. Afortunadamente, en los últimos 10 años se ha progresado bastante en la terapéutica de la leishmanaisis, y se cuenta con nuevas opciones como las formulaciones lipídicas de Anfotericina B, la miltefosina y la paromomicina. No obstante continúa siendo necesario fortalecer la farmacovigilancia de los tratamientos antileishmania, y monitorizar la aparición de resistencia a fármacos (Dujardin, 2006; WHO, 2010; Den Boer *et al*., 2011).

II.2- Presentación clínica

La mayoría de las infecciones cursan de manera asintomática, si bien el seguimiento a largo plazo indica que una pequeña proporción de estos individuos termina desarrollando una LV clínica.

En aquellos en los que se desarrolla la enfermedad tras la infección, el periodo de incubación puede variar de unos pocos días a un año, y el desarrollo generalmente es gradual. Los síntomas más comunes son fiebre, malestar, pérdida de peso y anorexia. Y los signos más frecuentes son esplenomegalia, en ocasiones asociada a hepatomegalia, caquexia y palidez de las membranas mucosas. El oscurecimiento de la piel es un signo encontrado típicamente en India (kala-azar en Hindi significa fiebre negra). A medida que la enfermedad progresa aparecen signos de malnutrición, siendo frecuente la aparición de infecciones concomitantes. Estos síntomas persistirán durante semanas o meses. Sin tratamiento, la mayoría de los casos pueden ser fatales, siendo las co-infecciones bacterianas, hemorragias masivas o anemia (causada por un estado inflamatorio persistente) las principales causas de muerte (Dujardin, 2006; Chappuis *et al*., 2007; WHO, 2010).

Las manifestaciones clínicas de la LV pueden ser diferentes en función de si esta es endémica, esporádica o epidémica (Cascio *et al*, 2002; WHO, 2010):

i) La LV endémica, en general, presenta un curso relativamente crónico. Cuando el agente causal es *L. infantum*, como en el sur de Europa, el norte de África, Asia central y oriental, y América, el grupo de edad más afectado son niños entre uno y diez años de edad. No obstante, en los últimos años la pandemia de SIDA y el aumento del uso de inmunosupresores han provocado que la mitad de los casos de LV de Europa ocurran en adultos. Cuando el agente causal es *L. donovani*, como en el este de África e India, la mayor incidencia se da en niños y adultos jóvenes.

ii) La LV esporádica suele ocurrir en población no autóctona de cualquier edad que entra en un foco endémico. Estos casos suelen ser agudos, y la enfermedad progresa rápidamente. Estos pacientes son más propensos a desarrollar formas complicadas de la enfermedad, como anemia hemolítica severa, daño renal y hemorragia mucosa.

iii) La LV epidémica ocurre generalmente en áreas de transmisión antroponótica, en este caso todos los grupos de edad son susceptibles, excepto aquellos que adquirieron inmunidad durante una epidemia previa. Se observan formas agudas de la enfermedad, y la tasa de mortalidad es generalmente elevada.

Se considera que determinados factores de riesgo pueden facilitar la progresión de la enfermedad tras la infección, como son la inmunodepresión (donde juegan un papel importante, en el contexto de la LV, la malnutrición y la infección por

VIH), la susceptibilidad genética y la existencia de otras infecciones concomitantes (Dujardin *et al*., 2006; WHO, 2010).

II.3- Respuesta inmune y patogénesis

El desarrollo de las diferentes formas clínicas de la leishmaniasis está determinado por la respuesta inmune del huésped y la especie de *Leishmania* implicada en la infección. En la mayor parte de los casos, la presencia del parásito desencadena una respuesta compleja por parte del huésped, en cuya fase inicial activa la respuesta inmune innata con la participación de células NK y producción de IL-12 que conduce a la respuesta inmune adaptativa gobernada principalmente por células colaboradoras de tipo 1 (Th1), en la que participan células dendríticas presentadoras de antígenos y células efectoras T CD4+ y CD8+ que secretan citoquinas pro-inflamatorias como la IL-12, IFN-γ y TNF-α, que a su vez activan los mecanismos leishmanicidas de los macrófagos infectados como son la producción de óxido nítrico y reactivos intermediarios del oxígeno (Murray *et al*., 2005). Esta respuesta genera una inmunidad celular sistémica y específica contra el parásito que permite controlar su multiplicación y diseminación, siendo capaz de mantener la infección subclínica y de proteger al individuo frente a futuras infecciones por *Leishmania*.

En otros casos, el parásito es capaz de evadir la respuesta inmune específica del huésped, afectando la capacidad presentadora de las células dendríticas, que evita el desarrollo de una respuesta Th1 específica, y también la capacidad leishmanicida de los macrófagos (MacMahon-Pratt y Alexander, 2004), lo que resulta en una inmunosupresión específica frente a *Leishmania* que lleva a la multiplicación y diseminación del parásito por diferentes órganos y tejidos y a la aparición de los síntomas clínicos característicos de la leishmaniasis visceral. La infección por *L. donovani* o *L. infantum* genera hiperplasia reticuloendotelial, afectando a bazo, higado, mucosa del intestino delgado, medula ósea, ganglios linfáticos y otros tejidos linfoides. Pudiéndose observar atrofia de estos órganos. Esto causa una reducción de la vida media de leucocitos y eritrocitos, provocando granulocitopenia y anemia. Puede alterarse la función hepática, disminuyendo el tiempo de producción de protrombina; esto, junto con la trombocitopenia puede generar hemorragia severa. También se puede observar hipoalbuminemia, que se asocia a la aparición de edema. Son también comunes la hipergammaglobulinemia y la activación policlonal de células B. En la fase avanzada la aparición de enfermedades concomitantes es frecuente, especialmente

neumonía, disentería y tuberculosis, que son la causa común de muerte en estos enfermos (WHO, 2010). Solo la quimioterapia efectiva es capaz de reducir la carga parasitaría en estos pacientes, lo que a su vez permite el desarrollo de una respuesta inmune tipo Th1 que coopera con el tratamiento en la recuperación completa del paciente con LV y es capaz de mantenerlo inmune frente a futuras infecciones.

II.3.1-Leishmaniasis dérmica post kala-azar

La leishmaniasis dérmica post-kala-azar (PKDL) es una complicación de la LV; se caracteriza por un sarpullido macular, maculopapular y/o nodular en pacientes que se han recuperado (o se están recuperando) de un episodio de LV. El sarpullido generalmente comienza alrededor de la boca, desde donde se propaga a otras partes del cuerpo en función de la severidad. Se observa principalmente in Sudán e India, ocurriendo tras el tratamiento de LV en el 50% y 5-10% de los casos respectivamente. El intervalo entre el episodio de LV y el PKDL varía de 0 a 6 meses en Sudán y de 2 a 3 años en India. Probablemente los enfermos de PKDL juegan un papel importante como reservorios entre epidemias de LV. El mecanismo exacto por el que se desarrolla el PKDL se desconoce, sin bien existe una creciente evidencia de que la patogénesis está mediada por la respuesta inmune (Zijlstra *et al*., 2003).

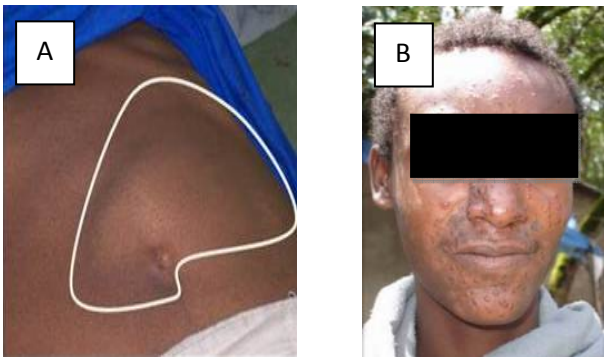

Figura 3. Esplenomegalia en paciente de LV (A). Formas nodulares de PKDL facial (B). (Autor: Endalamaw Gadisa)

II.4- Control de la leishmaniasis visceral

La transmisión de la leishmaniasis se mantiene en un complejo sistema biológico que implica huésped humano, parásito, vector y en algunas ocasiones un reservorio animal. Por tanto, cualquier estrategia de control requiere de un adecuado conocimiento del ciclo biológico en cada contexto epidemiológico. Se requerirá un

conocimiento exhaustivo de la dinámica de la enfermedad, y deberán combinarse distintas aproximaciones para romper la cadena de transmisión: diagnóstico, tratamiento y profilaxis, control del vector y del reservorio (si procede).

II.4.1- Diagnóstico

Las manifestaciones clínicas de la leishmaniasis no son específicas y pueden ser compartidas con las de otras infecciones sistémicas. Además, en función del área geográfica, habrá que prestar especial anterior al diagnóstico diferencial con otras patologías como la malaria o la esquistosomiasis. Por tanto, en la medida de lo posible, se precisa la confirmación mediante alguna prueba de laboratorio.

Diagnóstico parasitológico

La demostración del parásito tras crecimiento en cultivo, o mediante observación microscópica, de aspirados de médula ósea, bazo o ganglio linfático es la prueba confirmatoria de la infección y aun constituye el método *gold standard*. La observación microscópica de aspirados de bazo presenta una gran sensibilidad (93-99%), seguido de la de médula ósea (52-85%). El estudio del aspirado de ganglio linfático presenta una sensibilidad menor (52-65%), si bien en el este de África se ha reportado un buen rendimiento (Siddig *et al*., 1988; Zijlstra *et al*., 1992; Sundar y Rai, 2002; WHO, 2010).

Una alternativa que permite mejorar la sensibilidad en la detección del parásito es la reacción en cadena de la polimerasa (PCR). Además, la especificidad de la PCR puede adaptarse a necesidades particulares al utilizar como diana regiones hipervariables, variables o conservadas, y/o combinarse con otras técnicas de biología molecular, como la secuenciación, hibridación con sondas o digestión con endonucleasas de restricción. De este modo es posible caracterizar de manera rápida el parásito presente en la muestra en el nivel taxonómico de complejo, especie o incluso aislado individual, solventando los inconvenientes de las técnicas clásicas de identificación y caracterización (Reithinger y Dujardin, 2007). No obstante, una de las mayores aportaciones de la PCR al diagnóstico de la LV es su elevada sensibilidad en muestras de sangre periférica (70-100%), permitiendo realizar el diagnóstico a partir de muestras biológicas obtenidas mediante procedimientos menos invasivos (Antinori *et al*., 2007). Lamentablemente, su uso aún s emantiene restringido a hospitales de referencia y a centros de investigación (WHO, 2010).

Diagnóstico inmunológico

El diagnóstico inmunológico, ya sea a través de la evaluación de la respuesta humoral o celular, tiene gran interés en el caso de la LV. Pues es utilizado tanto en el diagnóstico de la enfermedad, como en la detección de infección asintomática o infecciones pasadas, lo que resulta de gran utilidad en estudios epidemiológicos.

Los métodos serológicos aprovechan la elevada producción de IgG anti-*Leishmania* durante la fase activa de la enfermedad. Se utilizan varios formatos, desde enzimoinmunoensayos y tests de aglutinación a inmuno-blots e inmunofluorescencia directa, considerándose esta última el método de referencia en el diagnóstico serológico de la LV. No obstante, no conviene olvidar que estos métodos no son capaces de distinguir entre infecciones activas o pasadas y que, en función del antígeno utilizado, podría obtenerse reacciones cruzadas con otros agentes infecciosos o enfermedades autoinmunes. Por otra parte, estos métodos pueden perder hasta un 50% de sensibilidad en el caso de enfermos coinfectados con el VIH (Cruz *et al*., 2006-a; Cruz *et al*., 2006-b). Existen dos métodos serológicos, el test de aglutinación directa (DAT) y una tira inmunocromatográfica rápida (rK39-ICT), que han sido desarrollados para poder ser aplicados sobre el terreno. El primero utiliza promastigotes completos y permite determinar el título de anticuerpos; el segundo utiliza el antígeno recombinante rK39 y es cualitativo. Ambos son baratos y fáciles de usar y han mostrado una sensibilidad (93-94%) y especificidad (95-97%) adecuadas en diversas áreas endémicas (Chappuis *et al*., 2006).

La respuesta inmune mediada por células T juega un papel crucial en el control de la infección por *Leishmania* (Murray *et al*., 1989). En infecciones asintomáticas o tras un tratamiento exitoso se desarrolla una respuesta de linfoproliferación específica que puede valorarse *in vivo* a través de la prueba de la leishmanina o test de Montenegro y ex *vivo* mediante un ensayo de linfoproliferación en placa. El test de Montenegro se encarga de medir una respuesta de hipersensibilidad retardada tras la inoculación intradérmica de una solución de promastigotes de *Leishmania* formolados (Weigle *et al.*, 1991). Los ensayos de linfoproliferación, al determinar la capacidad de proliferación linfocitaria frente a antígeno de *Leishmania*, proporcionan (al igual que el test de Montenegro) información sobre la capacidad del sistema inmune de montar una respuesta celular específica y protectora frente al parásito (Sacks *et al*., 1987). Los ensayos basados en la determinación de la respuesta celular frente a *Leishmania* no presentan utilidad a la hora de diagnosticar un episodio de LV, pero sí aportan

mucha información sobre la tasa de contacto con el parásito en población de área endémica (Alvar *et al*., 2007; Gidwani *et al*., 2009).

II.4.2- Tratamiento

El tratamiento debe suministrarse sólo a aquellos casos confirmados, y en ocasiones se aportará un suplemento nutricional o de rehidratación. De manera ideal, el tratamiento debe curar al paciente, reducir el riesgo de recaídas y de aparición de PKDL y reducir la transmisión. Durante las últimas siete décadas, los antimoniales pentavalentes han sido el tratamiento de primera opción para la LV, estando en la segunda línea la pentamidina y la anfotericina B deoxicolato. Afortunadamente, en los últimos diez años han aparecido nuevas opciones como las formulaciones lipídicas de anfotericina B, la miltefosina o la paromomicina. Actualmente se recomienda la combinación de fármacos, ya que ofrece varias ventajas: i) acorta el periodo de tratamiento, aumentando la adherencia al mismo, ii) se reduce la dosis, y por tanto disminuyen los efectos tóxicos y el precio, y iii) disminuye la posibilidad de aparición de cepas resistentes, aumentando así la vida útil de los fármacos disponibles (WHO, 2010).

II.4.3- Control del reservorio

En áreas donde la transmisión es antroponótica, las piezas clave en el control del reservorio serán un programa de detección activa de casos, vigilancia y la disponibilidad de un tratamiento eficaz. En áreas en que la LV es zoonótica (siendo el perro principal reservorio), es preciso determinar la distribución y frecuencia de la infección. Es importante considerar que no todos los perros infectados manifiestan síntomas de la enfermedad; por tanto, deben emplearse herramientas validadas en cada contexto epidemiológico a la hora de determinar la tasa de infección en los reservorios. Idealmente, los perros infectados deberían ser eliminados; si bien esta medida no ha resultado del todo eficaz en áreas donde se ha empleado durante largo tiempo, como en Brasil. Alternativamente, se debe potenciar el uso de insecticidas y/o repelentes, bien en forma de lociones o de collar (WHO, 2010).

II.4.4- Control vectorial

Un control vectorial efectivo tendrá un gran impacto en la transmisión del parásito, particularmente si se realiza en el hábitat doméstico y peridoméstico. Es de vital importancia conocer la ecoepidemiología de la LV en el área de intervención, así como la especie de vector (o vectores) implicada, su hábitat, rango de vuelo, preferencias alimentarias, lugares de reposo y refugio, así como su estacionalidad. Se puede proceder al control químico mediante el uso de insecticidas residuales de aplicación intradomiciliaria o el de telas mosquiteras impregnadas en insecticida (WHO, 2010).

III- LEISHMANIASIS VISCERAL EN ETIOPÍA

Se desconoce la carga total de LV en Etiopía, pero se estima una incidencia de aproximadamente 4 000 casos clínicos anuales (Alvar *et al*., 2008). También se considera emergente, pues se está propagando hacia áreas no endémicas dentro del país. Diferentes estudios epidemiológicos, realizados de forma esporádica, han identificado hasta 40 focos (Fig. 4). En la actualidad hay transmisión de LV en tres diferentes zonas ecológicas: en las tierras bajas del noroeste y suroeste, a menos de 1 500 metros sobre el nivel del mar, y en las tierras altas del la región centro-norte, a más de 1 800 metros sobre el nivel del mar.

El gran foco de LV de Humera y Metema se encuentra al noroeste del país, en las tierras bajas en la frontera con Sudán. Es el principal foco de LV de Etiopia, y acumula el 60% de los casos. Estas regiones son semiáridas, y el vector (*Phlebotomus orientalis*) se encuentra asociado a los suelos arcillosos y a los bosques de acacia. Desde principios de 1990 ha habido pequeños brotes en este foco debido a nuevos proyectos agrícolas a gran escala y a la ruta comercial desde Port-Sudan a Addis Abeba, que atraviesa los grandes focos de LV del sur de Sudan (Mengesha y Abuhoy, 1978; Maru 1979). Este foco tiene la mayor tasa de confección *Leishmania*/VIH, hasta un 40% de los pacientes con LV está infectado por el VIH (Alvar *et al*., 2008).

El 20% de los casos del país ocurre en la región suroeste, que engloba la sabana del suroeste de Etiopia, el área del lago Abaya, la meseta del río Omo, el área de Aba Roba y el valle de los ríos Segen y Woito (Ayele y Ali, 1984). En esta región los vectores implicados son *P. martini* y *P. celiae* que se encuentran en asociación con los nidos de termita (*Macrotermes termite*) (Gebre-Michael y Lane, 1996). En esta

zona, en las regiones de Afder y Liban, ha habido brotes de LV que han afectado regiones fronterizas de Kenia y Somalia (Marlet *et al*., 2003).

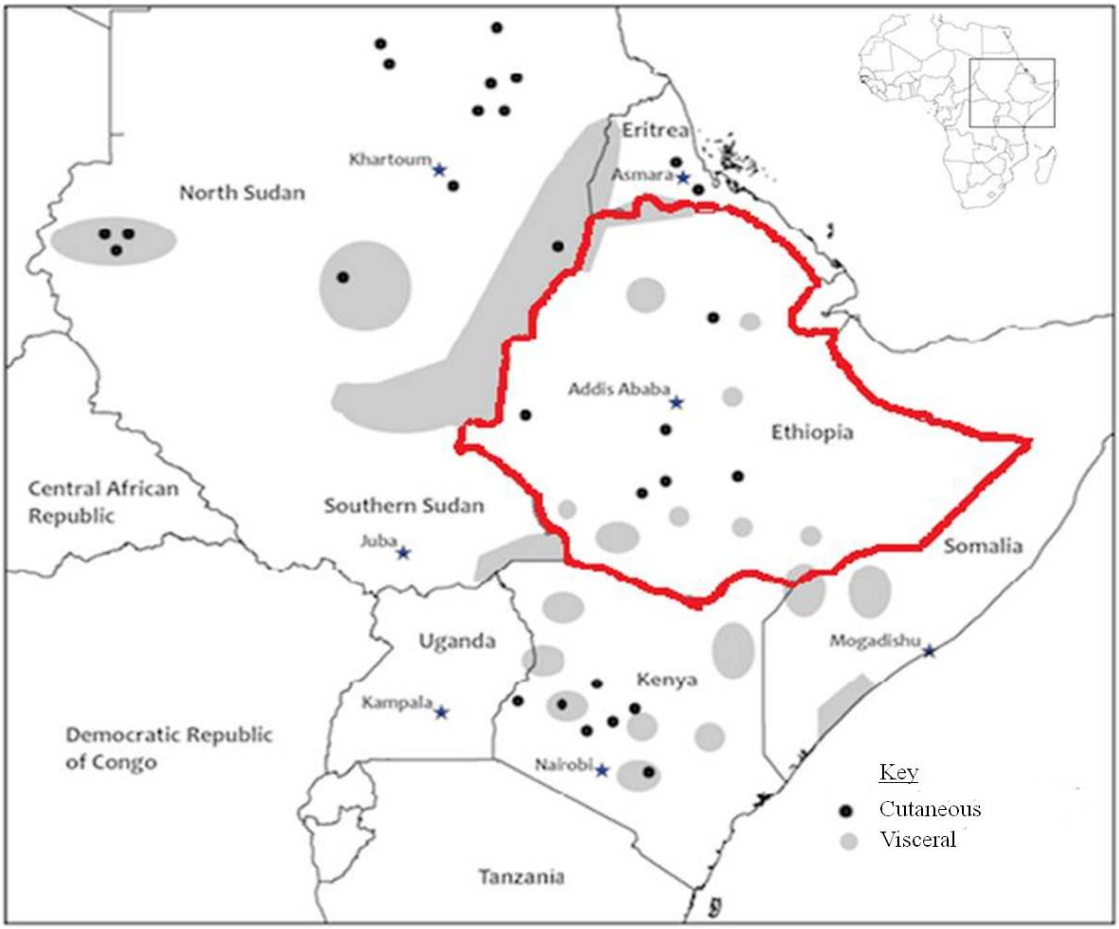

Figura 4. Distribución de la leishmaniasis en el Este de África

El foco más reciente se encuentra al nordeste del país, en los distritos de Libo Kemkem y Fogera (estado de Amhara), que se encuentran a 1800-2000 metros sobre el nivel del mar. En esta región, las actividades agrícolas han reducido la vegetación natural a grupos acacias dispersas por todo el territorio. Durante la estación de lluvias la mayoría del área queda inundada, durante la estación seca el suelo queda surcado por profundas grietas. Esta área se consideraba libre de LV hasta 2005, cuando Médicos sin Frontera-Grecia (MSF-G) reportó un brote en el distrito de Libo Kemkem (Alvar *et al*., 2007).

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V- EL PROYECTO *Visceral Leishmaniasis and Malnutrition in Amhara State, Ethiopia* DE LA FUNDACIÓN UBS-Optimus

El brote de LV en los distritos Libo Kemkem y Fogera resultó en la muerte de centenares de personas y puso en riesgo a más de 420 000 habitantes, por ello la OMS aconsejó su estudio y control. En respuesta a ello, el Centro Colaborador de la OMS para Leishmaniasis del Centro Nacional de Microbiología y el Centro Nacional de Medicina Tropical (ambos pertenecientes al Instituto de Salud Carlos III), y en colaboración con el Armauer Hansen Research Institute de Etiopía propusieron a la Fundación UBS-Optimus un proyecto para estudiar la leishmaniasis visceral desde un punto de vista nutricional inmunológico y parasitológico. Teniendo en cuenta que se trata de una región con una elevada tasas de malnutrición, y que la relación malnutrición-leishmaniasis visceral ha quedado ya demostrada; este proyecto pretende establecer el papel de la malnutrición en la epidemiología de la leishmaniasis visceral en este nuevo foco e identificar los factores socio-económicos están asociados a ella. De este modo se plantearán nuevas estrategias a la hora de prevenir la enfermedad.

Es en el marco de este proyecto donde se desarrolla el trabajo de la presente Tesis Doctoral.

Para mostrar mejor las características físicas del entorno, las condiciones de la población y las circunstancias en las que se ha realizado el trabajo, se ha incluido una galería de imágenes (ANEXO I) con fotos realizadas a lo largo del estudio que documentan sus diferentes fases y que pueden ayudar a comprender mejor el trabajo realizado.

VI- OBJETIVOS

1- Evaluación de herramientas diagnósticas para la detección de la infección asintomática por Leishmania (Artículo 1).

2- Determinar la prevalencia post brote de LV activa e infección asintomática para establecer si aún existe transmisión activa (Artículo 2).

3- Identificar factores de riesgo asociados a la infección (Artículo 3).

4- Estudiar la influencia de la malnutrición en la inmunidad adaptiva y su posible asociación a la susceptibilidad a infección y/o enfermedad (Artículo 4).

VII- ARTÍCULOS CIENTÍFICOS

The American Journal of Tropical Medicine and Hygiene

Usefulness of rK39-immunocromatrographic test, direct agglutination test, and leishmanin skin test to detect asymptomatic Leishmania infection in children from a new visceral leishmaniasis focus in Amhara State (Ethiopia)

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LRH: GADISA AND OTHERS

RRH: ASYMPTOMATIC LEISHMANIA INFECTION IN ETHIOPIA

Usefulness of rK39-immunochromatographic test, direct agglutination test, and leishmanin skin test to detect asymptomatic *Leishmania* infection in children from a new visceral leishmaniasis focus in Amhara State (Ethiopia)

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ABSTRACT

In areas where visceral leishmaniasis is anthroponotic, asymptomatic infected patients may play a role in transmission. Additionally, the number of asymptomatic patients in an endemic area will also provide information on transmission dynamics. Libo Kemkem and Fogera

districts (Amhara Region, Ethiopia) are now considered newly established visceral leishmaniasis endemic areas. In selected villages from these districts we have conducted a study to assess the usefulness of different approaches to estimate asymptomatic infection rate. Out of 639 participants rK39 immunochromatographic test was able to detect asymptomatic infection in 2% (13/639), direct agglutination test in 7.7% (49/639), and leishmanin skin test in 5.8% (37/623); the combined use of serological methods and leishmanin skin test allowed detecting asymptomatic infection in 12.8% (82/639). We conclude that the best option to detect asymptomatic infection is the combined use of both direct agglutination test and leishmanin skin test.

INTRODUCTION

Visceral leishmaniasis (VL) is a vector borne disease caused by members of the *Leishmania (Leishmania) donovani* complex, is endemic in 65 countries and, among them, Bangladesh, India, Nepal, Brazil, Sudan, and Ethiopia account for approximately 90% of the cases. The estimated annual incidence is 500 000 clinical cases with 59 000 associated deaths.[1,2] Poor populations are particularly affected by VL, which is considered as one of the ´most neglected diseases´.[3] In addition, VL is currently spreading and (re-)emerging in different areas of the world with increasing public health concern.[4]

In full-blown disease, VL is fatal if left untreated; and even with treatment the case fatality rate ranges from 4 to 10%.[5,6] In stable endemic areas clinical disease appears only in a fraction of those infected, while another fraction will not develop the disease and remain asymptomatic.[7] The prevalence of asymptomatic infection is quite different between and within different endemic countries, and the number of asymptomatic usually exceeds the number of symptomatic infections, though this ratio can vary from 0.4 : 1 to 50 : 1.[2]

Visualization of parasite amastigotes by microscopic examination of bone marrow, spleen or lymph node aspirates has been the Gold Standard method of VL diagnosis for many

years. However, as this procedure is based on an invasive sampling, its use for asymptomatic infection surveillance is not justified. Even more, in poor remote endemic areas the expertise and facilities required for these procedures may not be within reach. Thus procedures based on less invasive sampling, such as serology or leishmanin skin test (LST) seem to be more suitable for this purpose.

Although the detection of anti-*Leishmania* antibodies does not discriminate between current or past infection, serological methods have been used to assess asymptomatic infection in different VL endemic areas. These have been based either on the direct agglutination test (DAT), rK39-immunochromatographic test (rK39-ICT), enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT) or Western blot (WB).[8,9,10,11,12] The LST is a useful method to detect cell-mediated immunity against *Leishmania*, this test becomes positive after subclinical infection and, in this case, persists for much longer than anti-*Leishmania* antibodies; LST turns also positive within weeks-months after successful therapy against VL, indicating a healing or protective response.[13,14] This makes LST a valuable tool to detect exposure to *Leishmania* parasites in epidemiological surveys, and its usefulness to detect asymptomatic infection has been shown by different authors in different endemic areas.[11,15,16] In general LST would detect a higher proportion of asymptomatic infection than serology. However (given that serology and LST are based on different types of immune responses), in the absence of a Gold Standard, the combination of these two approaches would give a more realistic picture of the asymptomatic infection rate in a given endemic area.[17,18]

In areas where VL transmission is anthroponotic asymptomatic individuals may have a role as reservoirs, and even in areas where VL is zoonotic it is speculated that these individuals could also contribute to transmission.[7,19] Thus the assessment of the prevalence and distribution of asymptomatic cases would contribute to a better understanding of VL transmission, helping in control efforts.

In Ethiopia, VL is an endemic disease of increasing public health concern. It is estimated that thirty percent of the VL patients in Ethiopia are malnourished, while HIV co-infection affects 40% in the northwest of the country; both conditions are known to facilitate the spread of VL.[20,21] In addition to the classical foci in the northwest along the border with Sudan (Humera and Metema) and those in the south (Lake Abaya region, Omo river, and Aba Roba plains), the disease has recently spread to previously non-endemic areas, such as Libo Kemkem and Fogera districts (Amhara State) where a VL outbreak occurred in 2004--2005.[15] Thus VL is currently a priority in the public health agenda of the Amhara State Health Bureau; and there is a need to generate epidemiological data on VL in Amhara State. To support VL control, facilities for treatment, mobile teams for surveillance, community mobilization, and active case detection strategies have been established. In order to contribute to this initiative the UBS-Optimus Foundation granted the project entitled *Visceral leishmaniasis and malnutrition in Amhara State, Ethiopia*, which among its specific objectives aims to characterize nutritional, immunological, and parasitological aspects in the child population from this area; and it is in the frame of this project that we have explored the usefulness of rK39-ICT, DAT, and LST to detect asymptomatic *Leishmania* infection in children from different sub-districts of Libo Kemkem and Fogera. Furthermore, the detection of asymptomatic infection in children will give information about the status of VL transmission in this highland focus; additionally this will contribute to assess the magnitude of asymptomatic infection, which can help in early detection and treatment, thus contributing to decrease transmission as well as disease morbidity and mortality. The information obtained with this kind of studies can also be interesting in a scenario where HIV spreads to *Leishmania* endemic areas, helping to foresee new VL cases among asymptomatic individuals as a consequence of a reactivation of previous *Leishmania* infections after acquired HIV.[22]

MATERIALS AND METHODS

Study site, the study was conducted during May--July 2009 in the districts (*weredas*) of Libo Kemkem and Fogera (Amhara State, Ethiopia) (Figures 1 and 2). These are adjacent districts most affected by the outbreak of VL occurred in 2004-2005.[15] According to year 2009 census, the population was 198 374 (male 100 951 and female 97 423) and 226 595 (male 115 693 and female 110 902) for Libo Kemkem and Fogera respectively. The districts are located in a black cotton clay soil flat plain (1800--2000 meters above sea level). Human activities related to intensive cultivation of teff, maize, beans, oilseeds, rice and cotton, have reduced the natural vegetation to scattered clumps of acacia trees. Most of the area is flooded during the rainy season (July--September) and dried up during the dry season (November--May), resulting in deep cracks in the soil surface, which could turn into breeding sites for the putative vector *Phlebotomus orientalis*.[23,24]

Study population, population sampling was carried out by multi-staged cluster survey. Primary sampling units were sub-districts (*kebeles)* with high incidence of VL according to the 2008 register of the Addis Zemen VL Treatment Center: Agita, Bura, and Yifag from Libo Kemkem district and Dibasifatra and Rib Gebriel from Fogera district. Secondary sampling units were randomly selected villages (*gotts)* in each of the selected sub-districts. Third sampling units were randomly selected households in each of the villages. All children with a reported age between 4 and 15 yr living in the household at the time of the survey were eligible for the study, as long as they were asymptomatic and had no past history of VL.

Data collection, information on age, gender, residence, and clinical assessment was obtained for all participants by trained medical personnel (nurses and health officers) using pretested questionnaires and protocols.

Asymptomatic case definition, asymptomatic individuals were defined by a positive result in rK39-ICT, DAT or LST, and the absence of VL signs and symptoms (fever for > 2 weeks, in combination with either enlargement of spleen and/or liver, or weight loss).

Sample collection and storage, peripheral blood was collected in Na_2-EDTA tubes (SIGMA, UK) and immediately one drop was used for rK39-ICT and two drops were spotted on Whatman 3MM filter paper (Whatman International Ltd., England), filter papers were left to air dry and placed individually in sealed plastic bags. The plastic bags containing filter papers were kept in a chilled ice-box and sent on the same day to the Amhara State Regional Laboratory, where they were stored at 4 ºC for further DAT analysis.

Ethical considerations, the study was approved by the ethical review boards of Instituto de Salud Carlos III, Armauer Hansen Research Institute, and the Ethiopian National Ethical Review Committee. Support letters were obtained from the Amhara State and the different districts´ Health Bureaus. Parents/guardians gave written informed consent prior to the enrolment of their children in the study, and for children above 11 yr of age verbal assents were also obtained in addition to the consent of their parents /guardians.

Detection of anti-*Leishmania* antibodies, rK39-ICT (Kalazar Detect® Rapid Test, InBios International Inc., USA) was performed using one drop of blood and 3 drops of chasing buffer following the manufacturers' instructions. DAT with freeze-dried antigen (ITMA-DAT/VL, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium) was initially performed with the screening method according to the manufacturer's protocol. Blood samples giving a titer ≥ 1:3200 were considered positive.

Leishmanin skin test, LST was performed using *L. major* antigen (Leishmanin batch 123-2; Pasteur Institute, Iran). One hundred μL of the antigen were intradermally inoculated on the volar surface of the forearm with a 1 mL sterile syringe and disposable needle. The test was read 48 hr later by the ballpoint pen method. An induration with an average of two perpendiculars ≥ 5 mm was considered as positive.

Data analysis, infection prevalence was calculated using rK39-ICT, DAT, and LST results. The differences in infection prevalence between age group, sex, and location were

compared by the Fisher's exact and χ^2 tests. A p value < 0.05 was considered statistically significant. Data analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA,) and STATA version 10 (Stata Corp., College Station, TX, USA).

RESULTS

A total of 639 asymptomatic participants were included in the study: 331 were boys (51.8%) and 308 girls (48.2%), being the boy : girl ratio close to 1 : 1 in the two districts studied.

The mean age of the participants was 8.9 yr (SD: 3.2), without differences between both sexes. They were grouped according to their age in 3 different groups: group 1 (< 5 yr) consisted in 78/639 individuals (12.2%), group 2 (5--9 yr) in 310/639 (48.5%), and group 3 (10--15 yr) in 251/639 (39.3%).

Anti-*Leishmania* antibodies were detected in 13 out of 639 children (2.0%) by rK39-ICT and in 49/639 (7.7%) by DAT. Sixteen out of the 639 children (2.5%) initially tested by LST were lost for reading. This test returned a positive result in 37 out of 623 (5.9%).

Globally, 57 out of 639 children (8.9%) were found to be seropositive (rK39-ICT and/or DAT positive), and 82 out of 639 children (12.8%) were considered as infected (positive by rK39-ICT and/or DAT and/or LST). For the group of 623 children tested by the three methods we observed that 44/56 seropositive children had a negative LST result. While 25 out of 37 LST positive children were seronegative. A detailed description of the performance of these three methods in the group of 623 children is given in Table 1.

The mean age in those with asymptomatic infection was 10.0 yr (SD: 2.9). Analysis by age group revealed a positive association between asymptomatic infection and age, children between 10--15 yr being the group with a higher asymptomatic infection rate (18.7%; p = 0.005). This finding was common to all test employed.

A strong association was also found with regard to gender, with infection in boys being higher than in girls (17.2% *vs* 8.1%; p = 0.001), differences in asymptomatic infection rate by age group and gender is provided in Table 2.

The highest prevalence of asymptomatic infection was found in the selected villages from Bura (27.9%) and the lowest in those from Agita (3.6%).

A detailed description of the results obtained by the three methods employed by gender, age and location is given in Table 3.

DISCUSSION

The present work reveals the presence of asymptomatic *Leishmania* infection among 4-15 yr old children from the districts of Libo Kemkem and Fogera, in the new VL focus of Amhara State, Northwestern Ethiopia. The observed overall asymptomatic infection rate was 12.8% (82/639); determined by the combination of serological methods, which detected 57/639 seropositive individuals (8.9%), and LST, detecting 37/623 (5.9%) positive individuals. As proposed initially the combination of serology and LST allowed a wider detection of asymptomatic infection.

The discordances observed between serology and LST were expected. Both LST and serology have been used to assess exposure to *Leishmania*, irrespective of disease presentation, and are frequently used in epidemiological studies in *Leishmania* endemic areas.[9,25,26] Nevertheless, comparison of LST positivity and seroprevalence rates is complicated due to the different type of immune response detected by each test. LST measures a delayed type hipersensitivity reaction to *Leishmania* and relies on an *in vivo* cellular immune response to *Leishmania* antigens, while seropositivity is the result of a significant level of *Leishmania*-specific antibodies in the peripheral blood, which is based on a humoral immune response. The two differentiated groups of LST-positive/seronegative and LST-negative/seropositive children observed in our study are in agreement with the lack of association between LST and serology.

LST positivity appears later after infection and seems to be a sign of protective immunity against VL, while seropositivity is considered a marker of more recent infection and has been related to disease progression.[27,28] However, a recent sero-epidemiologic study in Bihar (India) observed low disease conversion rate in asymptomatic DAT positive individuals. [29]

Different studies in VL endemic areas have shown that LST detects a higher *Leishmania* infection rate in asymptomatic individuals than serological approaches. [8,11,26,30,31,32] In contrast our study shows that the infection rate obtained with serology is higher than that obtained with LST, 8.9% *vs* 5.9% using DAT and rK39-ICT and 7.6% *vs* 5.9% using DAT alone. Given that VL has recently been reported in our study area this finding can be associated to the longer time needed for the development of a LST positive response compared to sero-conversion. If we consider that LST positive conversion is the result of a repeated exposure to natural infection this would also explain why LST positivity is higher in the older age group.

We have observed a high discordance between DAT and rK39-ICT in our work (Table 1). Eight individuals with a positive rK39-ICT result were negative by DAT, while 54 DAT positive individuals presented a negative rK39-ICT result; and only 4 individuals presented a positive result by both serological methods. Although Ritmeijer and others reported a lower sensitivity of rK39-ICT in Sudan, a meta-analysis on the performance of DAT and rK39-ICT for active VL diagnosis concluded that both tests have a similar level of sensitivity.[33,34] Additionally a recent study in Libo Kemkem evaluating the performance of DAT and two different rK39-ICT brands indicated that either approach is suitable for VL diagnosis in this area of Ethiopia.[35] However our study population is asymptomatic and higher positivity rates have been reported for DAT *vs* rK39-ICT when these methods are used to assess asymptomatic infection in other VL endemic areas.[7] Given that performance of a serologic test can depend on the stage of the disease/infection, the lower performance of rK39-ICT can be explained by its ability to detect antibody response against only a single antigen (rK39), while DAT relies on

the detection of antibodies against a wide range of *Leishmania* antigens (the whole freeze-dried promastigote).[36] Another explanation can be based on the nature of the tests. As proposed by Ter Horst and others, antibodies detected by rK39-ICT could be less able to react in a rapid reaction than in the overnight incubation used for DAT.[37]

Although DAT has shown better performance to detect asymptomatic infection in our study population, the presence of 8 individuals with a positive rK39-ICT result but negative for DAT merits attention. A possible explanation for this could be the different volume of blood tested by each method. While in our study rK39-ICT was performed on one drop of blood, DAT was performed on a 5 mm disk punched out from a spot of two drops of blood on a filter paper, which can be considered a lower amount of blood. Additionally, it was observed by Zijlstra and others that an rK39-based test (though in ELISA format) could detect asymptomatic infection earlier than DAT.[38]

The increase of asymptomatic infection rate with age was consistent with an endemic focus of VL, with a marked increase for the age group of 5--9 yr onwards. In addition, the presence of asymptomatic infection in individuals aged less than 5 yr is also consistent with the presence of active transmission, in spite of the low VL incidence situation reached after the outbreak.[6]

The present study indicates the appropriateness of combining serology (particularly DAT) and LST to obtain a consistent picture of the asymptomatic infection rate in a VL endemic area. This work also indicates that after the 2004--2005 VL outbreak active transmission is still happening in the villages studied, and also that *L. donovani* transmission can potentially be established in Ethiopian highlands (1800--2000 meters above sea level) which are commonly considered free of VL.

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Figure 1: Location of the study area

Figure 2: Location of the sub-districts on which the study was performed (grey background)

Table 1: Detailed description of the performance of rK39-ICT, DAT and LST in the group of 623 children

rK39-ICT	**DAT**	**LST**	**n/N (%)**
Negative	Negative	Negative	542/623 (87.0)
Negative	Negative	**Positive**	25/623 (4.0%)
Negative	**Positive**	Negative	37/623 (6.0%)
Positive	Negative	Negative	5/623 (0.8%)
Positive	**Positive**	Negative	2/623 (0.3%)
Positive	Negative	**Positive**	3/623 (0.5%)
Negative	**Positive**	**Positive**	7/623 (1.1%)
Positive	**Positive**	**Positive**	2/623 (0.3%)

Table 2: Rate of asymptomatic infection by gender and age (N = number of children tested by variable group; n = number of infected children per variable group; % = percentage of infected children by variable group)

	Rate of asymptomatic infection		
Age group (yr)	Boys [n/N (%)]	Girls [n/N (%)]	Both sexes [n/N (%)]
< 5	1/34 (2.9)	2/44 (4.5)	3/78 (3.8)
5--9	21/147 (14.3)	11/163 (6.7)	32/310 (10.3)
10--15	35/150 (23.3)	12/101 (11.8)	47/251(18.7)
Total	57/331 (17.2)	25/308 (8.1)	82/639 (12.8)

Table 3: Asymptomatic infection rates detected by the three different tests and their combination displayed by gender, age and location (N = number of children tested per variable group; n = number of positive children by each test per variable group; % = percentage of positive children by each test per variable group; *Figures for LST are related to a 623 children population)

	TESTS				
VARIABLES	rK39-ICT n (%)	DAT n (%)	LST* n (%)	Seropositive n (%)	Infected n (%)
WHOLE POPULATION (N = 639)	13 (2.0)	49 (7.7)	37 (5.9)	57 (8.9)	82 (12.8)
GENDER					
Boys (N = 331)	9 (2.7)	33 (9.9)	27 (8.3)	40 (12.1)	57 (17.2)
Girls (N = 308)	4 (1.3)	16 (5.2)	10 (3.3)	17 (5.5)	25 (8.1)
Age group (yr)					
< 5 (N = 78)	1 (1.3)	3 (3.8)	1 (1.3)	3 (3.8)	3 (3.8)
5--9 (N = 310)	5 (1.6)	23 (7.4)	8 (2.6)	26 (8.4)	32 (10.3)
10--15 (N = 251)	7 (2.8)	23 (9.1)	28 (11.4)	28 (11.1)	47 (18.7)
SUBDISTRICT					
Agita (N = 139)	1 (0.7)	5 (3.6)	0 (0.0)	5 (3.6)	5 (3.6)
Bura (N = 147)	5 (3.4)	22 (14.9)	24 (16.4)	24 (16.3)	41 (27.9)
Dibasifatra (N = 140)	6 (4.3)	4 (2.8)	6 (4.4)	9 (6.4)	13 (9.3)
Rib Gebriel (N = 144)	1 (0.7)	13 (9.0)	7 (4.9)	14 (9.7)	18 (12.5)
Yifag (N = 69)	0 (0.0)	5 (7.2)	0 (0)	5 (7.2)	5 (7.2)

Figure 1

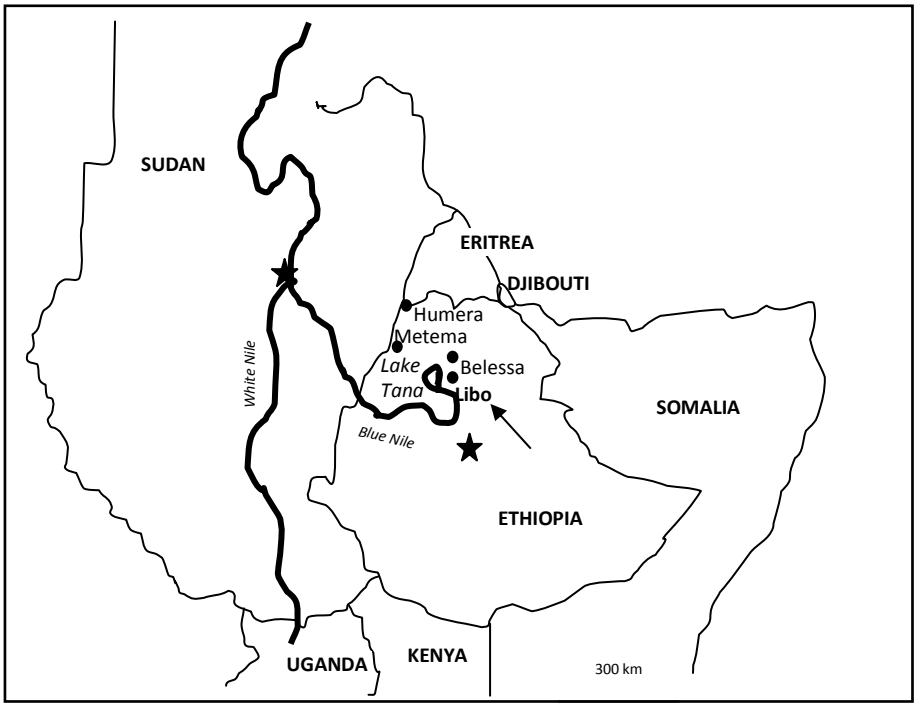
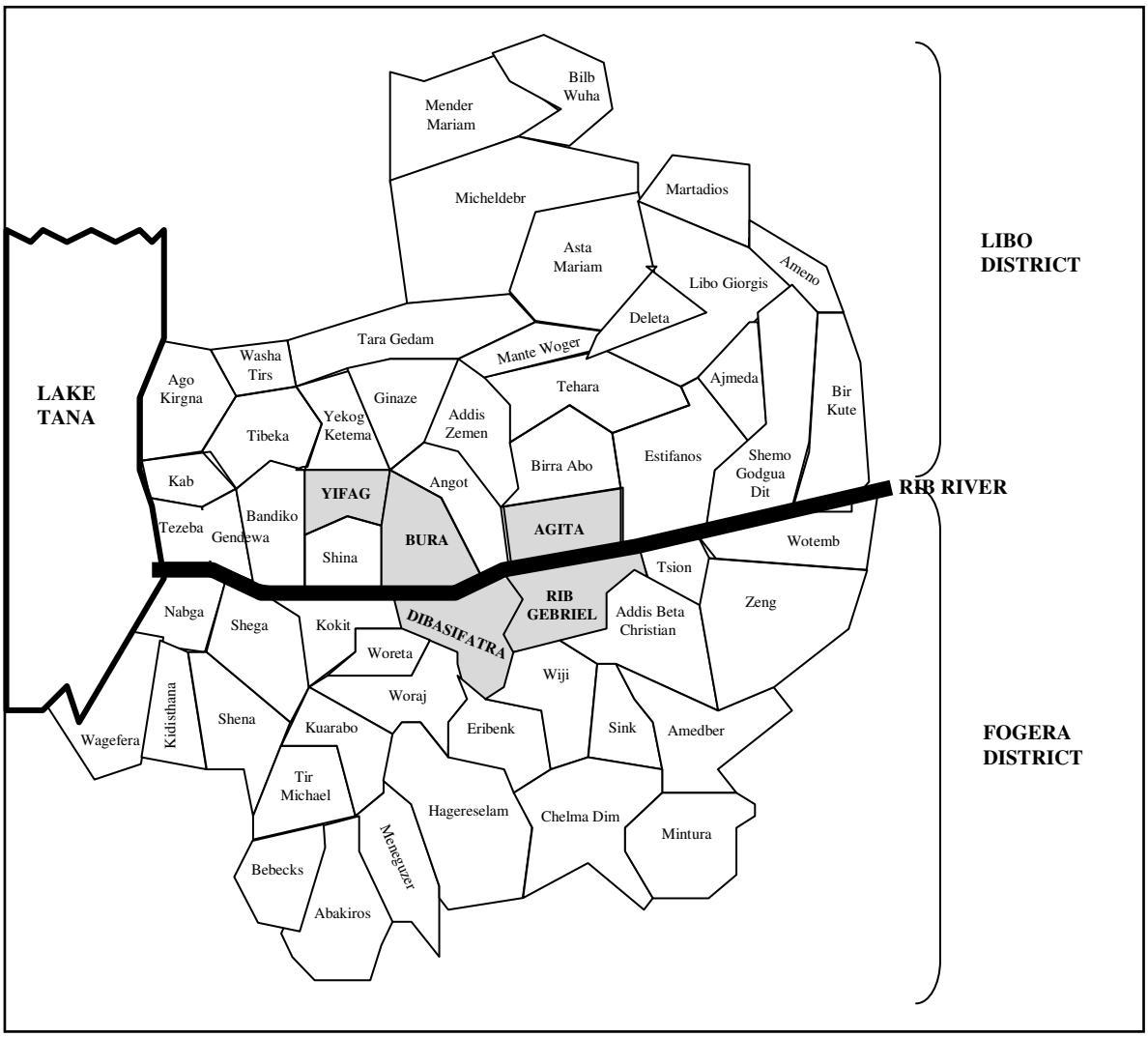


Figure 2



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Low prevalence of Leishmania infection in post-epidemic areas of Libo Kemkem, Ethiopia

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LRH: SORDO ET AL.

RRH: PREVALENCE OF LEISHMANIA INFECTION IN LIBO KEMKEM

Low prevalence of *Leishmania* infection in post-epidemic areas of Libo Kemkem, Ethiopia

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ABSTRACT

In Libo Kemkem (a district of the Amhara region, Ethiopia), no cases of kala-azar had ever been reported until 2005, when an outbreak occurred. Over one third of those affected were children under 15 years of age. The aim of the present study was to determine the

prevalence of *Leishmania* infection in children aged 4--15 years. A cross-sectional survey was conducted in 2009. Sampling was performed in a multi-staged cluster survey. A total of 386 children were included in the study. The overall prevalence of *Leishmania* infection (DAT- and/or rK39-ICT- and/or LST-positive subjects) in this population was then estimated. Only one case of active disease was encountered. The overall prevalence of infection was 1.02% (95% CI: 0--4.54), being higher among boys and in children older than 12 years. The results suggest that the conditions responsible for the outbreak no longer reign. However, active surveillance remains necessary.

East Africa has suffered a marked increase in the number of cases of visceral leishmaniasis (VL) or kala-azar over the last two decades, probably due to a combination of demographic and climatic changes.[1,2]

The areas traditionally regarded as endemic for VL in Ethiopia lie in the north-west (bordering Sudan) and south of the country.[3,4] Libo Kemkem district (*wereda*) is located in the highlands of the Amhara region in northwestern Ethiopia at an altitude of 1800--2000 m. No cases of kala-azar had ever been declared in this area until 2005. Earlier, in 2004, the Amhara Regional Health Bureau reported a 5-fold increase in crude mortality rates in the Libo Kemkem district, attributing this to an outbreak of drug-resistant malaria. However, in May 2005 an outbreak of kala-azar was determined to be the culprit.[5] The epidemiological background - a few cases over a 1-year period followed by an explosive increase - was consistent with the rapid emergence of the disease in a population with little pre-existing immunity.[5] By December 2007, 2543 patients with kala-azar had been treated by *Médecins sans Frontières*.[6] More than one third were children under 15 years of age, for whom a fatality rate of over 3% was reported.[6] The rapid spread of the disease between 2004 to 2007 suggested that transmission would not be easy to control.[5] Before the Libo Kemkem outbreak there was no epidemiological surveillance system for leishmaniasis in Ethiopia, making it difficult to determine whether the epidemic between 2004--2007 was an outbreak due to a recent introduction of the parasite or, as suggested by Herrero *et al.*,[6] the parasite was endemic to the area but in low numbers.

The aim of the present study was to determine the prevalence of *Leishmania* infection in children aged 4--15 years from Libo Kemkem, four years after the outbreak.

A cross-sectional survey was conducted between May and July 2009, as part of the project *Visceral Leishmaniasis and Malnutrition in Amhara State, Ethiopia*, funded by the UBS-Optimus Foundation. Sampling was undertaken as part of a multi-staged cluster survey.

The Primary sampling units were randomly selected sub-districts (*kebeles*) of Libo Kemkem that, according to the records of *Médecins sans Frontières-Greece* held at the Addis Zemen Health Centre, had reported at least one case of VL during the 2004--2007 epidemic. These were selected taking into account their size according to a recent census.[7] The secondary sampling units were randomly selected villages (*gotts)* in each of the selected sub-districts. The tertiary sampling units were households randomly selected from an updated census for each village. All children between 4 and 15 years of age residing in these household were tested. A total of 386 children were included in the study.

Ethical clearance was obtained from the review boards of the *Instituto de Salud Carlos III*, the Armauer Hansen Research Institute, and the Ethiopian National Ethical Review Committee. Parents/guardians gave written, informed consent prior to the enrolment of their children in the study. For children over 11 years of age, verbal assent was obtained in addition to the consent of their parents or guardians.

Each participant was clinically assessed by health professionals for any complaint of fever lasting longer than two weeks, weight loss, and the presence of splenomegaly and lymphadenopathy, in order to determine the presence of any active infection. All children were tested using the leishmanin skin test (LST), the rK39-immunochromatographic test (ICT) (Kalazar Detect® Rapid Test, InBios International Inc., Seattle, WA), and the direct agglutination test (DAT) (ITMA-DAT/VL, Institute of Tropical Medicine, Antwerp, Belgium). Sociodemographic data were recorded using pre-tested questionnaires. The rK39-ICT test was performed immediately after blood sampling, according to the manufacturer's instructions. The DAT test was performed on blood-impregnated filter paper using freeze-dried antigen. The screening method followed the manufacturer's protocol; titres of ≥1:3200 were deemed positive. Leishmanin skin testing was performed using *L. major* antigen (Leishmanin batch 123-2; Pasteur Institute, Tehran, Iran), as previously described.[8,9]

The overall prevalence of *Leishmania* infection (DAT- and/or rK39-ICT- and/or LST-positive) in the population was then calculated. Prevalence rates were expressed in percentages with 95% confidence intervals (95% CI). Stata v.10.1 software was used to perform all statistical analyses. Data were weighted according to selection probabilities and analysed using the Stata v.10.1 complex samples procedures, which takes into account sample clustering.

One hundred and ninety nine of the 386 children (50.9%) were girls. The mean age of the participants was 8.9 years (SD 3.03); 44.76% of the children were under 8 years of age, and 33.08% between 8 and 11 years.

Only one case of active VL was found, which returned positive results for both rK39-ICT and DAT. Nine children were DAT-positive only; four of them had suffered kala-azar previously. However, five children that previously had VL showed negative results for both rK39-ICT and DAT. None of the children returned a positive LST result.

The overall prevalence of infection (DAT and/or rk39 positive) was 1.02% (95% CI: 0--4.54). The prevalence among boys was higher (1.78%; 95% CI: 0--7.98, vs. 0.3%; 95% CI: 0--1.31 in girls). The greatest prevalence was recorded in children older than 12 years (2.56%; 95% CI: 0--10.54), followed by those between 8 and 11 (0.82%; 95% CI: 0--3.53); the under 8 years subgroup showed the lowest prevalence (0.49%; 95% CI: 0--2.59). However, these prevalence values were not statistically different to one another (Table 1).

To the best of our knowledge, these are the first *Leishmania* infection prevalence data for Libo Kemkem since the 2004--2007 epidemic. Higher prevalence rates for similar populations have, however, been reported for other regions of Ethiopia.[10,11]

The permanence of Leishmanin skin test reactivity is thought to depend on latent infection and the continuous exposure to biting, *Leishmania*-carrying sand flies;[10,17] a decrease of these vectors might explain the absence of positive results in the present study

population. This would also explain the reduction in the number of VL cases reported in this area.[6] Some authors report a natural conversion rate from positive to negative of 14.8% to 9.3% in other settings in Ethiopia.[12] Nevertheless, the absence of positive results is noteworthy, especially in comparison with previous results in the same age range (23.6%),[5] though in the latter study rapid assessment via convenience sampling was undertaken.

The present study also used DAT and rK39 ICT detection of infection, methods that reveal the presence of anti-*Leishmania* antibodies appearing early after infection. The combined use of these methods, which has performed well in VL diagnosis in this area,[13] yielded just 1% prevalence. It should be remembered that this result is only for 4--15 year-old and not for the whole population. However, although the prevalence of *Leishmania* infection increases with age,[10] the presence of only one active case in the study population and the low prevalence even among the older children, suggests a low prevalence for the general population.

The present results appear to indicate that the conditions that provoked the kala-azar epidemic in the study region no longer reign. The parasite may have been introduced by migrant agricultural labourers who, returning to their villages after completing seasonal work on the border of Sudan,[14] acted as a reservoir of the causal parasite – a hypothesis put forward at the time of the epidemic. However, the available evidence indicates that the affected population was not made up simply of migrant workers, and there is no evidence that any such migration has ever ceased. This suggests that, as well as an increase in the size of the human reservoir, some change in the vector population must have occurred. Libo Kemkem lies at an altitude of 1800--2000 m, beyond that at which phlebotomes are normally found. However, as described by other authors,[15] changes in the temperature or relative humidity could have encouraged their increased presence. In other scenarios such changes have been attributed to global warming.[15,16] It should be noted, however, that the response to the

outbreak included the establishment of centres where specific treatment could be received. The clearance of patients' infections would have led to a reduction in the seroprevalence rate.

Until 2004, no case of leishmaniasis had ever been reported in Libo Kemkem. The very low prevalence in the study population may suggest that the district may be experiencing a pre-epidemic status similar to that seen prior to the outbreak. Efforts to identify areas of high prevalence and then focus control efforts in these places might be wiser than blanket control of the entire district. However, the doubts surrounding the reasons for the outbreak means vigilance with respect to the impact of possible climate changes should be considered; such changes might encourage new outbreaks.

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Table 1: Prevalence of *Leishmania* infection

	Sample		Prevalence of *Leishmania* infection (rK39- and/or DAT-positive)				
	N[a]	%[b]	N[a]	%[b]	95% CI[b]	OR (95% CI)	p
Overall	386		10	1.02	0-4.54		
Sex							
Girls	199	50.9	2	0.3	0-1.31	Ref.	
Boys	187	49.1	8	1.78	0-7.98	5.94 (0.38-93.84)	0.291[c]
Age							
<8 years	169	44.76	2	0.49	0-2.59	Ref.	
8-11 years	132	33.08	3	0.82	0-3.53	1.675 (0.1-29.12)	
>11 years	85	22.16	5	2.56	0-10.54	5.228 (0.43-63.4)	0.39[c]

[a]Unweighted
[b]Weighted
[c]Fisher

PLoS Neglected Tropical Diseases

Factors Associated with Leishmania Asymptomatic Infection in Highland Northern Ethiopia

--Manuscript Draft--

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Abstract:	Background: In northern Ethiopia the prevalence of visceral leishmaniasis is steadily rising posing an increasing public health concern. In order to develop effective control strategies on the transmission of the disease it is important to generate knowledge on the epidemiological determinants of the infection. Methodology/Principal Findings: We conducted a cross-sectional survey using a multi staged stratified cluster sampling on high incidence sub-districts of Amhara state, Ethiopia. The survey included a socio-demographic, health and dietary questionnaire and anthropometric measurements. We performed RK39-ICT and DAT serological tests in order to detect anti-Leishmania antibodies and carried out Leishmanin Skin Test (LST) using L. major antigen. Logistic regression models were used. Of the 605 children surveyed 61 children were positive to infection (10.1%). The individual variables that showed a positive association with infection were increasing age, being male and sleeping outside [odds ratios (95% CI): 1.12 (1.02, 1.23), 2.06 (1.10, 3.82) and 2.10 (1.15, 3.85) respectively] and in relation to the household: increasing number of people living in the house, past history of VL in the family, living in a straw roofed

	house and if the family owned sheep [OR (95% CI): 1.26 (1.06, 1.50), 2.66 (1.44, 4.92), 2.35 (1.24, 4.45) and 3.25 (1.52, 6.98) respectively]. The presence of dogs in the house [OR (95% CI): 0.44 (0.23, 0.85] showed an inverse association. Conclusions/Significance: Behavioural patterns like sleeping outside are determinant in the transmission of the infection in this area. Protective measures should be implemented against these identified risk activities. Results also suggest a geographical clustering of the infection and a transmission within the homestead, human to human, but more studies are needed on the behaviour of the vector to clarify possible entomological interventions related to housing conditions. The role of domestic animals in transmission needs to be studied further before giving any recommendation.
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To the PLOS Neglected Tropical Diseases Editors:

It is my pleasure to send you enclosed a paper entitled "**Factors associated with *Leishmania* asymptomatic infection in highland northern Ethiopia**" for consideration as an article in the PLOS Neglected Tropical Diseases.

Visceral leishmaniasis prevalence is steadily rising in northern Ethiopia, posing a public health challenge in the region. In two highland (1800-2000 mts above sea level) provinces of Amhara state an outbreak occurred in 2005 that has ever since transited into an endemic focus with sustained transmission.

The role of asymptomatic infected individuals in the control and prevention of the disease is important, as they can and act as reservoirs for new infection or become ill if immunosuppression occurs. Factors associated with infection can differ from disease correlates, and may also change in relation to the vector behaviour in the local environment.

We find important to disseminate the results of our study not only towards the reinforcement of the program control developed by the Amhara Regional Health Bureau, but also in order to contribute to the scientific community with the epidemiological determinants of *Leishmania* asymptomatic infection in an environment that, to the best of our knowledge, has not been described before. We certainly consider the PLOS Neglected Tropical Diseases the best vehicle for it.

All authors have contributed to the conception and design of the work, the acquisition of data, or the analysis of the data in a manner substantial enough to take public responsibility for it. All authors believe the manuscript represents valid work and have reviewed the final version of the manuscript and approve it for publication. None of the authors had any conflict of interest.

Finally, the material included in the manuscript is our original work and has not been published before, nor has been submitted for publication elsewhere.

Looking forward to hearing from you,

Yours sincerely,

Estefanía Custodio

Title: Factors associated with *Leishmania* asymptomatic infection in highland northern Ethiopia

Short Title: *Leishmania* asymptomatic infection correlates

Authors: Estefanía Custodio[1], Endalamaw Gadisa[2], Luis Sordo[3,4] , Israel Cruz[5], Javier Moreno[5], Javier Nieto[5], Carmen Chicharro[5], Abraham Aseffa[2], Zelalem Abebe[6], Hailu Tsegaye[2] and Carmen Cañavate[5]

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ABSTRACT

Background: In northern Ethiopia the prevalence of visceral leishmaniasis is steadily rising posing an increasing public health concern. In order to develop effective control strategies on the transmission of the disease it is important to generate knowledge on the epidemiological determinants of the infection.

Methodology/Principal Findings: We conducted a cross-sectional survey using a multi staged stratified cluster sampling on high incidence sub-districts of Amhara state, Ethiopia. The survey included a socio-demographic, health and dietary questionnaire and anthropometric measurements. We performed RK39-ICT and DAT serological tests in order to detect anti-*Leishmania* antibodies and carried out Leishmanin Skin Test (LST) using *L. major* antigen. Logistic regression models were used. Of the 605 children surveyed 61 children were positive to infection (10.1%). The individual variables that showed a positive association with infection were increasing age, being male and sleeping outside [odds ratios (95% CI): 1.12 (1.02, 1.23), 2.06 (1.10, 3.82) and 2.10 (1.15, 3.85) respectively] and in relation to the household: increasing number of people living in the house, past history of VL in the family, living in a straw roofed house and if the family owned sheep [OR (95% CI): 1.26 (1.06, 1.50), 2.66 (1.44, 4.92), 2.35 (1.24, 4.45) and 3.25 (1.52, 6.98) respectively]. The presence of dogs in the house [OR (95% CI): 0.44 (0.23, 0.85] showed an inverse association.

Conclusions/Significance: Behavioural patterns like sleeping outside and herding the cattle are determinant in the transmission of the infection in this area. Protective measures should be implemented against these identified risk activities. Results also suggest a geographical clustering of the infection and a transmission within the homestead, human to human, but more studies are needed on the behaviour of the vector to clarify possible entomological interventions related to housing conditions. The role of domestic animals in transmission needs to be studied further before giving any recommendation.

AUTHORS SUMMARY:

Visceral leishmaniasis is a vector borne disease that can be fatal if left untreated. Its prevalence is steadily rising in northern Ethiopia posing a public health challenge in the region. We conducted a study on the factors associated to asymptomatic infection in Amhara state, where little is known about *Leishmania* transmission. Sleeping outside and herding the cattle were identified as risk activities, so the implementation of preventive measures towards them is recommended. Our results also suggested a transmission within the homestead, human to human, but more entomological studies are needed in order to clarify the vector's behaviour in the area. Individuals living in houses that owned sheep were more likely to be infected and those living in houses with dogs seemed to be protected. This last result differs from the direct association between dog's ownership and the clinical form of the disease found in a case control study carried out in this same area. These conflicting results add up to the debate found in the literature regarding the role of domestic animals in the transmission of *Leishmania* in different regions of the world. No specific recommendation should be given until the exact role of the domestic animal in the transmission cycle is clearly understood.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a neglected vector-borne parasitic disease that manifests with irregular bouts of fever, substantial weight loss, weakness, hepatosplenomegaly and pancytopenia, and that is fatal if left untreated [1]. It has an estimated annual incidence of 500 000 clinical cases with 50 000 associated deaths and 2 357 000 disability-adjusted life years lost. It is mainly concentrated in few major foci and the East African *L.donovani* focus is the second largest, with the highest incidence in Ethiopia and the Sudan [2].

VL is caused by protozoan parasites of the *Leishmania donovani* species complex transmitted to human and animal hosts by the bite of phlebotomine sand flies. It has already been determined that large numbers of individuals in endemic areas are infected with the parasite but do not develop any signs or symptoms of the disease. The reported ratio of asymptomatic infections to VL clinical cases varies widely from 4:1 in Kenya [3] to 50:1 in Spain [4]. This variation is presumed to reflect differences in parasite virulence and host population characteristics, and may also depend on the study designs and on the tests used to define asymptomatic infection [1].

The methods more widely used in order to assess asymptomatic infection in the field are a) serological assays that detect anti-*Leishmania* antibodies based either on the direct agglutination test (DAT) or the rK39-immunochromatographic test (rK39-ICT) and b) Leishmanin Skin Test (LST) that measures cell-mediated immunity against *Leishmania* [5,6].

It is important to generate knowledge on the factors associated with asymptomatic infection for the optimal design and implementation of prevention and control strategies of VL, as asymptomatically infected individuals can harbor latent parasite and act as reservoirs for new infection or become ill if immunosuppression occurs [7,8].

In northern Ethiopia, the prevalence of VL is steadily rising posing an increasing public health concern. The region has recently experienced epidemics in previously unaffected areas [2]. In 2005, a kala-azar outbreak occurred in the district of Libo Kemkem in Amhara regional state, described by Alvar et al [9] . A case control study was conducted there in 2007 to evaluate the risk factors associated with the clinical form of the disease [10]. However, and as it has been previously stated, the epidemiological determinants of clinical VL and sub clinical infection are not necessarily the same [11].

Thus, the aim of this study is to describe the determinants of asymptomatic leishmanial infection among the villages with high incidence of VL in Libo Kemkem and Fogera in order to provide further information that will help the Amhara regional health authorities to develop effective strategies to control the transmission of the disease.

MATERIAL AND METHODS

Study area and population

The study was conducted during May-July 2009 in the districts (*weredas*) of Libo Kemkem and Fogera (Amhara State, Ethiopia). These are adjacent districts most affected by the outbreak of VL that occurred in 2005 [9]. In 2009, the population numbered 198 374 and 226 595 in Libo Kemkem and Fogera, respectively. The economic status of the population is uniformly low. The districts are located in a black cotton clay soil flat plain (1800-2000 meters a.s.l.). Human activities related to intensive cultivation of *teff*, maize, beans, oilseeds, rice and cotton, have reduced the natural vegetation to scattered clumps of acacia trees. Most of the area is flooded during the rainy season (July-September) and dried up during the dry season (November-May), resulting in deep cracks in the soil surface, which could turn into breeding sites for the putative vector *Phlebotomus orientalis* [12,13].

Study design

Population sampling was carried out by a multi-staged cluster survey. Primary sampling units were sub-districts (*kebeles*) with high incidence of VL according to the 2008 register of the Addis Zemen VL Treatment Center: Bura, Yifag Akababi and Agita from Libo Kemkem and Sifatra and Rib Gebriel from Fogera. Secondary sampling units were randomly selected villages (*gotts*) in each of the selected sub-districts. Third sampling units were randomly selected households in each of the villages. All children with reported age between 4 and 15 years living in the household at the time of the survey, and who had not previously suffered VL, were included in the study, as long as they were

asymptomatic (absence of VL signs and symptoms: fever for > 2 weeks, in combination with either enlargement of spleen and/or liver, or weight loss).

Data collection

A blood sample was taken from the selected children in order to detect anti-*Leishmania* antibodies. The rK39-ICT (Kalazar Detect® Rapid Test, InBios International Inc., USA) was performed following the manufacturers' instructions. DAT with freeze-dried antigen (ITMA-DAT/VL, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium) was carried out on blood-impregnated filter paper following the screening procedure according to the manufacturer's instructions. Titers ≥1:3200 were considered positive.

Leishmanin Skin Test was carried out using *L. major* antigen (Leishmanin batch 123-2; Pasteur Institute, Iran). The test was read 48 hours later by the ballpoint pen method. An induration with an average of two perpendiculars ≥5 mm was considered as positive.

All children were measured and weighted according to standard WHO procedures [14]. Wasting was defined as Body Mass Index (BMI) for age <-2 Z scores, and stunting as Height for Age < -2 Z scores according to the 2006 WHO Growth Standards for children ≤ 5 years and to the 2007 WHO Growth Reference for children > 5 years respectively [15].

Care providers of the children were interviewed by trained health professionals using standardized questionnaires that included questions on demographics, household characteristics, child health, dietary habits and VL prevention behaviours. The questionnaires used were pretested and translated into Amharic, the local language.

Data Analysis

The primary outcome of interest was *Leishmania* asymptomatic infection defined as a positive result either in rK39-ICT, DAT or LST. The serological tests and the LST measure different types of the immune response and are thus not likely to produce the same results. Therefore we created two secondary outcomes: a) Seropositive: positive to rK39-ICT and/or DAT irrespective of the LST result and b) LST Positive: positive to LST irrespective of the serostatus.

We attempted to estimate the factors associated with asymptomatic infection and then to isolate the factors associated with the seropositivity and LST positivity by making independent analysis for the three outcomes described above.

Multivariate analysis to examine the socio-economic, behavioural, nutritional and dietary predictors of asymptomatic infection indicators were carried out using logistic regression models adjusting for potential confounding variables that were significant in the univariate analysis (carried out using bivariate logistic regression). Variables included in the model as numeric have the p value for trend and the Odds Ratios (OR) and Confidence Intervals (CI) described in the text and the OR (CI) for categories detailed in the tables. Multivariate models included all variables for which adjusted estimates are presented.

A p value less than 0.05 was considered statistically significant.

Data analysis was performed using AnthroPlus v1.02 (WHO, Geneva, Switzerland), and SPSS version 18.0 (SPSS Inc., Chicago, Illinois, USA).

Ethical considerations

The study was approved by the ethical advisory boards of Instituto de Salud Carlos III in Spain and the Armauer Hansen Research Institute and the Ethiopian National Ethical Review Committee in Ethiopia. Support letters were obtained from the Amhara Regional State and the district Health Bureaus. All parents/guardians gave written informed consent prior to the enrolment of their children in the study. Assent was also obtained from children $\geq$ 11 years of age.

RESULTS

All the *gotts* selected were rural. Around 90% of the households were headed by males and in more than 99% of the households the occupation of the head was related to farming activities (farmer, labourer, cotton worker, etc.). Ninety eight per cent of the households reported owning land. The mean size of land owned by a household was 1.6 Ha (range 0.01 – 8 Ha). Only 6.4% of the households owned more than 3 Ha. More than 95% of the households reported owning some type of domestic animals, mainly cows (89.8%), chicken (59.2%)

and sheep (23.6%). Thirty two per cent of the households had radio and only 0.3% had access to electricity.

A total of 605 children were surveyed (51.1% boys and 48.9% girls) with a mean age of 8.8 (3.2 SD). Sixty one children (10.1%) had asymptomatic infection, of which 38 (6.3%) were seropositive and 33 (5.6%) LST positive. There was a wide variation in the number of asymptomatically infected children according to *gotts*, with the *gotts* in Bura *kebele* presenting the highest frequencies (see Table 1).

Among the children, 245 (40.7%) were found to be stunted and 130 (21.6%) were wasted. Only 4.6% had consumed animal food source products the day before the interview.

Unadjusted analysis of infection

Table 2 and Table 3 summarize the individual and household characteristics that showed significant association in the univariate analysis with any of the outcomes previously described.

The individual factors that showed a positive association with asymptomatic infection were*:* increasing age, male sex, being wasted, sleeping outside and herding cattle; and the household characteristics: increasing number of people in the household, having a past history of VL in the family, living in a household straw roofed house and owning sheep three years before

and at the time of the survey. On the other hand, owning dogs three years before and at the time of the survey showed an inverse association with asymptomatic infection.

Sleeping outside at any time of the day was the only individual variable that showed a direct association with seropositivity besides increasing age and being male. In terms of household characteristics, those that presented a positive association were increasing number of people in the household, living in a family with past history of VL and if the household owned sheep three years before and at the time of the survey. In the opposite direction, the number of cattle owned by the family at the time of the survey showed an inverse association.

The individual variables that showed a direct association with LST positivity were the same as those for asymptomatic infection, except for wasting. The use of bed net by a child, although not statistically significant suggested an inverse relationship (p=0.07). In terms of household conditions, increasing number of people in the family and straw roof showed a positive association with a positive LST, and the increasing number of cattle owned by the family and the presence of dogs three years before and at the time of the survey showed a negative one.

Adjusted analysis

281 Table 4 shows the results of the multivariate logistic regression for
282 asymptomatic infection, seropositivity and LST positivity.
283
284 The individual variables that kept in the model positively associated with
285 asymptomatic infection after adjustment were: increasing age, being male and
286 sleeping out at any time [OR (95% CI): 1.12 (1.02, 1.23), 2.06 (1.10, 3.82) and
287 2.10 (1.15, 3.85) respectively]. In terms of household characteristics: increasing
288 number of people living in the household, past history of VL in the family, living
289 in a straw roofed house and if the family owned sheep three years before and at
290 the time of the survey [OR (95% CI): 1.26 (1.06, 1.50), 2.66 (1.44, 4.92), 2.35
291 (1.24, 4.45) and 3.25 (1.52, 6.98) respectively]. And with an inverse and
292 significant association, the presence of dogs in the house three years before
293 and at the time of the survey [OR (95% CI): 0.44 (0.23, 0.85].
294
295 Being male and increasing age were the only two individual variables that
296 kept direct and significant association with seropositivity after adjustment [OR
297 (95% CI): 2.69 (1.26, 5.77) and 1.12 (1.00, 1.25) respectively]. Living in a family
298 with past history of VL and if the household owned sheep three years before
299 and at the time of the survey also showed a positive association [OR (95% CI):
300 3.66 (1.84, 7.28) and 2.43 (1.02, 5.78) respectively]. And in the opposite
301 direction, the number of cattle remained inversely associated with seropositivity
302 [OR (95% CI): 0.85 (0.74, 0.99)].
303
304 The individual factors positively and significantly associated with LST
305 were sleeping outside and herding the cattle [OR (95% CI): 3.14 (1.33, 7.39)

and 3.80 (1.04, 13.89) respectively]; and in terms of household conditions the only one positively associated with it was increasing number of people in the household [OR (95% CI): 1.29 (1.03, 1.63)] and in the opposite direction the presence of dogs three years before and at the time of the survey [OR (95% CI): 0.29 (0.13, 0.67)]

No significant association was found between any of the outcomes analysed for asymptomatic infection and stunting; sex, age or education of the head of the household; household electricity, radio or land owning, floor and walls construction material and condition; number of meals or consumption of animal source food products; number of bed nets in the household, house spraying status; the existence of an animal shed, animal dung or a termite mound near the house; and number of chicken owned by the household.

DISCUSSION

The prevalence of asymptomatic infection found in our study sample as well as the factors associated with it differed depending on the outcome variable used for the analysis.

The discordances observed between serology and LST have been discussed elsewhere [16-18]. The last LST screening in the area was conducted in 2005 as part of the outbreak assessment, and the prevalence of LST positivity was considerably higher than in our study, 34% for men and 26% for women [9]. This discrepancy is consistent with the fact that the cited study

was conducted in a different population age range (0.7 to 60 years old) and at the peak of the epidemic in an area that has since transited into an endemic focus with a sustained low transmission, as described recently by Herrero et al (Herrero, 2009). The strong variation in the prevalence of asymptomatic infection among clusters highly endemic for VL is congruent with the spatial clustering observed in other studies of asymptomatic infection [19,20] and of clinical VL cases [21,22]. Notably, Bura, the kebele where the 2005 outbreak started, has maintained the highest prevalence ever since [9].

The increase of asymptomatic infection rate with age observed in our study area is consistent with an endemic focus of VL, in spite of the low VL incidence situation reached after the outbreak [23]. The permanence of LST reactivity is thought to be a consequence of cumulative past exposure, thus prevalence typically rises with age [24]. The positive association between *Leishmania* infection and older age, as well as with male sex, has also been related to activities like cattle herding or sleeping outside, that imply an increased potential exposure to the sand fly vector, and that are culturally specific to male adolescents and male adults [19,25]. Our results would support this hypothesis, as cattle herding and sleeping outside were also identified in our study population as risk factors for asymptomatic infection and had previously been identified as risk factors for VL in South Ethiopia [26] and North Ethiopia (in our study area) as well [10]. The greater exposure to sand flies when herding livestock can be associated with the moving near and/or far away from homesteads at dusk and down when the sand flies are active [27] and also with an increased proximity to acacia trees. Resting under acacia trees has

been identified as a risk factor for VL in our study area [10]. Among our surveyed population, 82% of the herder children reported resting under acacia trees while herding. Acacia trees are thought to be diurnal resting sites for *Phlebotomus orientalis,* the described potential vector of the disease in the area [12,13].

Poor nutritional status has been associated with a higher risk of developing visceral leishmaniasis in other studies [28-31] although to the best of our knowledge, an association with asymptomatic infection has not yet been described. In our findings wasting appeared as a risk factor for asymptomatic infection but only in the unadjusted analysis, so we can not conclude there is association between nutritional status, measured by anthropometry, and asymptomatic infection in our study population.

The use of bed net appeared to be protective but did not reach a significant association with any of the infection outcomes used in the analyses, which is in agreement with other studies in relation to asymptomatic infection [18,32]. The protective effect of bed net use against visceral leishmaniasis remains unclear, with variable results depending on the setting and study [11,33]. The lack of protective effect found in our study could be associated with the net condition, nature of utilization and impregnation status, conditions that were not assessed in our survey.

A larger family size may appear as a risk factor for asymptomatic infection based on attraction of sand flies by greater biomass, as it was

described for the risk of VL in this same area [10]. Other studies have also shown a positive association with seropositivity and previous VL cases contact [3,20,34], supporting the hypothesis of transmission within the homestead, human to human. This hypothesis has been further strengthened by the failure of other studies to relate the household clustering of infection and disease to characteristics of the house or the surroundings [3,28]. It is important to highlight that the increased likelihood of asymptomatic infection among children with a past VL case in the family remained significant only for seropositivity and not for LST positivity, in concordance with findings of Bern et al in Bangladesh [32]. In one study conducted in Kenya, it was found that the association between LST positivity and previous VL cases in the family was significant only for women and young children, suggesting that women were exposed in and around the house and males, in addition, exposed elsewhere [35]. We tested this hypothesis by conducting separate analyses for male and female populations but results did not vary (data not shown).

Living in a straw roofed house versus an iron thatched one was the only house characteristic associated with asymptomatic infection. It could be related to socioeconomic status or to the potential of straw roofs to provide resting places for the sand fly that would increase its survival and abundance. Mud-type houses have been identified as risk factors for VL or asymptomatic infection before and have been associated with better living conditions or with the vector preference for mud crack walls for breeding and resting [22,26,36,37]. However, regarding *P. orientalis* more studies are needed, as the

few extant studies in the literature point out to an exophagic behaviour of the vector, ill suited with this hypothesis [38].

In relation to domestic animals the associations found are conflicting. Owning sheep increases the risk of asymptomatic infection but the presence of dogs and an increasing number of cattle owned by the family minimizes it.

The positive correlation of disease and the presence of sheep has already been described [19,22], and has been explained by the greater biomass and the accumulation of animal dung that may be attractive to the sand flies, drawing the vectors into closer association with humans.

The role of cattle in the transmission of the disease remains unclear and has been subject of a recent review [11]. Several studies coincide with our results in the protective effect of cattle towards VL [21,22,39] and the possible explanation given is that the number of cattle acts as wealth indicator and/or that there is a potential vector blood preference for these domestic animals that will exert a zooprophylactic effect. *P. orientalis* blood preference for cattle versus humans has already been shown in other areas of Ethiopia, supporting this idea [38]. Interestingly, this protective effect was seen in relation to seropositivity, but not to LST positivity, in concordance with findings in Bangladesh by Bern et al [32], where increased cattle density was found to be protective against VL and DAT positivity but was identified as a risk factor for LST positivity.

The negative correlation between the presence of dogs and asymptomatic infection is in contrast to the finding of owning dogs as a risk factor for VL in this same area [10]. The protective role of dogs against asymptomatic infection had been described in Brazil [34], but other studies have not found any significant association with either asymptomatic infection or VL [20,22].

Our study had a number of limitations. One of them is the cross sectional nature of the study, which limits the making of causal inferences between the analysed factors and the infection. Another limitation is that the study subjects were not tested for HIV, which may alter the results. However, adult HIV incidence in Amhara state was 0.32 in 2007 [40] (theoretically lower for children below 15 years old) so although it is possible that a subset of our cases may have had HIV it would be a small number and we are confident it would not alter our general conclusions.

CONCLUSIONS

Selected behavioural and housing factors were associated with higher rates of asymptomatic infection and these can be the focus of interventions. Protective measures should be implemented against the identified risk activities such as herding cattle or sleeping outside. More studies are needed on the behaviour of the *P.orientalis* in the area in order to clarify possible entomological interventions related to housing conditions. Given the contrasting results found in our study and in the literature, and the complex nature of the

relationship between disease transmission and domestic animals, the exact role of domestic animals in transmission needs to be studied further before any intervention is recommend in this regard.

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Table 1: *Leishmania* asymptomatic infection, seropositivity and LST positivity prevalence by *gott* in Amhara state, Ethiopia.

				Asymptomatic infection*		Seropositivity*			LST positivity*±	
Name of cluster/Gott	**Kebele**	**Wereda**	**N**	**n**	**%**	**n**	**%**	**N**	**n**	**%**
FOGERIE MENDER	Agita	Libo Kemkem	32	0	0	0	0	32	0	0
FUAT FUAT	Agita	Libo Kemkem	34	2	5.9	2	5.9	31	0	0
GILGEL TERARA	Agita	Libo Kemkem	32	0	0	0	0	29	0	0
MELAGUD	Agita	Libo Kemkem	35	0	0	0	0	35	0	0
MEDROGE	Bura	Libo Kemkem	46	11	23.9	10	21.7	46	7	15.2
MEHAL-EGZIABHERAB	Bura	Libo Kemkem	31	11	35.5	1	3.2	31	10	32.3
MENTA-WARKA	Bura	Libo Kemkem	29	8	27.6	6	20.7	29	2	6.9
QUARA	Bura	Libo Kemkem	34	5	14.7	1	2.9	33	4	12.1
GULTOCH	D. Sifatra	Fogera	34	4	11.8	3	8.8	34	2	5.9
LAHADA	D. Sifatra	Fogera	35	2	5.7	1	2.9	34	1	2.9
RAS DIBA	D. Sifatra	Fogera	34	0	0	0	0	33	0	0
SIFATRA	D. Sifatra	Fogera	30	4	13.3	3	10	27	1	3.7
AMAGA	Rib Gebriel	Fogera	30	3	10	3	10	30	0	0
DENBOCH	Rib Gebriel	Fogera	35	2	5.7	1	2.9	35	1	2.9
GICHOCH	Rib Gebriel	Fogera	35	2	5.7	2	5.7	32	2	6.2
GOMBEL	Rib Gebriel	Fogera	32	4	12.5	2	6.3	32	3	9.4
ANSHA	Yifag Akababi	Libo Kemkem	33	2	6.1	2	6.1	33	0	0
BATA	Yifag Akababi	Libo Kemkem	34	1	2.9	1	2.9	33	0	0
Total			605	61	10.1	38	6.3	589	33	5.6

*As defined in the Materials and Methods section
LST=Leishmanin Skin Test
± A total of 589 children had the LST performed.

Table 2: Individual variables associated with *Leishmania* asymptomatic infection in children. Unadjusted analysis.

Factor	N (%)	Asymptomatic infection *		Seropositivity*		LST positivity*±	
		Positive n (%)	Odds ratio§ (95% CI)	Positive n (%)	Odds ratio§ (95% CI)	Positive n (%)	Odds ratio§ (95% CI)
Age							
<5 years	77 (12.7)	2 (2.6)	Reference	2 (2.6)	Reference	1 (1.3)	Reference
5 to 9 years	295 (48.8)	21 (7.1)	2.87 (0.66, 12.53)	15 (5.1)	2.01 (0.45, 8.98)	8 (2.7)	2.16 (0.27, 17.53)
10 to 15 years	233 (38.5)	38 (16.3)	7.31 (1.72, 31.05)	21 (9.0)	3.71 (0.85, 16.22)	24 (10.6)	8.87 (1.58, 66.69)
p for trend			<0.0001		0.03		0.002
Sex							
Girl	296 (48.9)	18 (6.1)	Reference	18 (6.1)	Reference	10 (3.4)	Reference
Boy	309(51.1)	43 (13.9)	2.50 (1.40, 4.41)	43 (13.9)	2.85 (1.36, 5.98)	23 (7.6)	2.30 (1.07, 4.92)
p global			0.002		0.006		0.032
Wasting¶							
No	472 (78.4)	47 (10.0)	Reference	29 (6.1)	Reference	25 (5.4)	Reference
Yes	130 (21.6)	14 (10.8)	1.10 (0.58, 2.05)	9 (6.9)	1.14 (0.52, 2.46)	8 (6.2)	1.15 (0.51, 2.62)
p for trend			0.05		0.11		0.14
Sleeps outside							
No	366 (60.6)	21 (5.7)	Reference	16 (4.4)	Reference	8 (2.2)	Reference
Yes	238 (39.4)	40 (16.8)	3.32 (1.93, 5.08)	22 (9.2)	2.23 (1.14, 4.34)	25 (10.7)	5.21 (2.31, 11.77)
p global			<0.0001		0.018		<0.0001
Herds the cattle							
No	250 (41.3)	12 (4.8)	Reference	10 (4.0)	Reference	3 (1.2)	Reference
Yes	355 (58.7)	49 (13.8)	3.17 (1.65, 6.10)	28 (7.9)	2.05 (0.98, 4.31)	30 (8.5)	7.54 (2.27, 24.91)
p global			0.001		0.06		0.001
Uses bed net							
No	365 (60.3)	41 (11.2)	Reference	21 (5.8)	Reference	25 (6.9)	Reference
Yes	240 (39.7)	20 (8.3)	0.72 (0.41, 1.26)	17 (7.1)	1.25 (0.64, 2.42)	8 (3.4)	0.47 (0.21, 1.07)
p global			0.25		0.51		0.072
Overall	605						

*As defined in the Materials and Methods section

LST=Leishmanin Skin Test

± Only 589 children had the LST performed.

§OR obtained by logistic regression

¶Defined as Body Mass Index for Age Z score < -2 SD based on the 2006 WHO Growth Standards for children ≤ 5 y and on the 2007 WHO Growth Reference for children > 5 y

Table 3: Household characteristics associated with *Leishmania* asymptomatic infection in children. Unadjusted analysis.

Factor	N (%)	Asymptomatic infection*		Seropositivity*		LST positivity*±	
		Positive n (%)	Odds ratio§ (95% CI)	Positive n (%)	Odds ratio§ (95% CI)	Positive n (%)	Odds ratio§ (95% CI)
People in the family							
< 5 people	201 (33.3)	13 (6.5)	Reference	10 (5.0)	Reference	5 (2.6)	Reference
5-7 people	257 (42.6)	23 (8.9)	1.42 (0.70, 2.88)	14 (5.4)	1.10 (0.48, 17.53)	14 (5.6)	2.24 (0.79, 6.33)
> 7 people	145 (24.0)	24 (16.6)	2.87 (1.41, 5.85)	14 (9.7)	2.04 (0.88, 4.73)	13 (9.0)	3.71 (1.29, 10.64)
p for trend			0.003		0.05		0.03
Past history of kala azar in the family							
No	442 (73.2)	34 (7.7)	Reference	18 (4.1)	Reference	22 (5.1)	Reference
Yes	162 (26.8)	27 (16.7)	2.40 (1.39, 4.12)	20 (12.3)	3.32 (1.71, 6.45)	14 (7.5)	1.37 (0.65, 2.91)
			0.002		<0.0001		0.4
House roof material							
Corrugated iron	223 (36.9)	32 (14.3)	Reference	19 (8.5)	Reference	18 (8.4)	Reference
Straw	382 (63.1)	29(7.6)	2.04 (1.19, 3.47)	19 (5.0)	1.78 (0.92, 3.44)	15 (4.0)	2.19 (1.08, 4.34)
p global			0.009		0.09		0.03
Number of cattle owned by the household							
No cattle	62 (10.2)	7 (11.3)	Reference	6 (9.7)	Reference	4 (6.9)	Reference
Less than 10 cattle	502 (83.0)	50 (10.0)	0.87 (0.37, 2.01)	30 (6.0)	0.59 (0.24, 1.48)	27 (5.5)	0.78 (0.26, 2.33)
More than 10 cattle	41 (6.8)	4 (9.8)	0.85 (0.23, 3.11)	2 (4.9)	0.48 (0.09, 2.49)	2 (4.9)	0.69 (0.12, 3.97)
p for trend			0.1		0.041		<0.0001
Household owns sheep							
No	462 (76.4)	38 (8.2)	Reference	27 (5.8)	Reference	18 (4.0)	Reference
Yes	143 (23.6)	23 (16.1)	2.56 (1.30, 5.04)	11 (7.7)	2.38 (1.04, 5.45)	15 (10.5)	2.36 (0.98, 5.67)
p global			<0.0001		0.039		0.056
Household owns dogs							
No	273 (45.1)	35 (12.8)	Reference	22 (8.1)	Reference	21 (7.8)	Reference
Yes	332 (54.9)	26 (7.8)	0.58 (0.34, 0.98)	16 (4.8)	0.58 (0.29, 1.12)	12 (3.1)	0.46 (0.22, 0.95)
p global			0.04		0.11		0.035
Overall	605						

*As defined in the Materials and Methods section
LST=Leishmanin Skin Test
± Only 589 children had the LST performed.
§OR obtained by logistic regression

Table 4: Factors associated with *Leishmania* asymptomatic infection in children. Adjusted analysis.

Factor		Asymptomatic* infection Odds ratio (95% CI)	Seropositivity* Odds ratio (95% CI)	LST positivity* Odds ratio (95% CI)
Child age				
	<5 years	Reference	Reference	Reference
	5 to 9 years	2.61 (0.57, 12.02)	1.99 (0.43, 9.16)	2.03 (0.44, 9.44)
	10 to 15 years	5.62 (1.24, 25.45)	3.57 (0.79, 16.07)	4.20 (0.89, 19.49)
	p for trend	0.014	0.041	0.16
Child sex				
	Girl	Reference	Reference	Reference
	Boy	2.06 (1.10, 3.82)	2.69 (1.26, 5.77)	1.34 (0.59, 3.03)
	p global	0.02	0.011	0.48
Child sleeps outside				
	No	Reference	"	Reference
	Yes	2.10 (1.15, 3.85)		3.14 (1.33, 7.39)
	p global	0.016		0.009
Child herds the cattle				
	No			Reference
	Yes	"	"	3.80 (1.04, 13.89)
	p global			0.044
People in the family				
	< 5 people	Reference	"	Reference
	5-7 people	1.54 (0.72, 3.32)		1.45 (0.69, 3.05)
	> 7 people	3.04 (1.32, 7.00)		3.01 (1.37, 6.60)
	p for trend	0.009		0.029
History of past kala azar in family				
	No	Reference	Reference	
	Yes	2.66 (1.44, 4.92)	3.66 (1.84, 7.28)	"
	p global	0.002	<0.0001	
House Roof Material				
	Corrugated iron	Reference		
	Straw	2.35 (1.24, 4.45)	"	"
	p global	0.009		
Number of cattle				
	No cattle		Reference	
	Less than 10 cattle	"	0.38 (0.14, 1.04)	"
	More than 10 cattle		0.28 (0.05, 1.57)	
	p for trend		0.035	
Household owned sheep 3 years before and at the time of the survey				
	No	Reference	Reference	
	Yes	3.25 (1.52, 6.98)	2.43 (1.02, 5.78)	
	p global	0.002	0.046	
Household owned dogs 3 years before and at the time of the survey				
	No	Reference		Reference
	Yes	0.44 (0.23, 0.85)		0.29 (0.13, 0.67)
	p global	0.014		0.003

*As defined in the Materials and Methods section
LST=Leishmanin Skin Test
± Only 589 children had the LST performed.
§OR obtained by logistic regresssion

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Effect of childhood protein energy malnutrition on the immunological status of children from the new Visceral Leishmaniasis focus in Libo Kemkem (Amhara State, Ethiopia)

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Abstract

Childhood Protein Energy Malnutrition (PEM) is a major public health problem in developing countries. Epidemiologic studies documented strong association between PEM and risk of child death and/or severity of infections. However, the mechanisms that govern the relationship between PEM and infectious diseases are multiple and not well described. The present study examined the effect of PEM on selected immunological players implicated in the infection by *Leishmania* parasites. Our data showed significantly lower total count of white blood cells, lymphocytes and T cell subpopulations (CD4+ and CD8+) in severely malnourished children. PBMCs from the severely malnourished children produced significantly low or no cytokine after 24 hours of stimulation with PHA. Moderately malnourished children produced significantly high level of PGE2 as compared to the non-malnourished children. Non-malnourished children showed high level of cytokines in the serum, while no detectable levels of any of the cytokine assayed were found in severely malnourished children and only IL-4 and IL-10 were detected in the serum of moderately malnourished children. The DAT positive non-malnourished children produced significantly high level of IFN-γ and IL-10 as compared to the moderately and severely malnourished children. The detection of low levels of serum IFN-γ and relatively high levels of IL-4 and IL-10 in the moderately malnourished children might indicate a Th2 bias in this group

probably associated to the high level of PGE2 production. In conclusion, severe malnutrition affected not only the absolute number of the immune cells in children but also their functional activity, an adverse effect that might be mediated by overproduction of biochemical factors induced by malnutrition like PGE2.

INTRODUCTION

Childhood Protein Energy Malnutrition (PEM) is a major public health problem in developing countries. In Africa it is estimated that 45% of the children suffer from malnutrition [1]. There is a strong relationship between malnutrition, infection, and infant mortality. Human epidemiologic studies have documented that PEM is a primary cause of immunodeficiency and a major determinant of both progression and severity of infections caused by different pathogens [2-4]. Children with mild and moderate malnutrition present a reduced cell mediated and humoral immune function [5] which can make them more susceptible to infections. A recent meta-analysis showed that HIV prevalence is high in malnourished children in sub-Saharan Africa, and they are at a significantly increased risk of mortality [6]. Zachariah et al, observed that moderate to severe malnutrition is a risk factor associated with TB [7]. A strong association of giardiasis and PEM has also been reported [8]. The specific anti-IgG response to *Plasmodium falciparum* was found to be significantly lower in severely stunted children as compared to non stunted [9].

Visceral leishmaniasis (VL) or kala-azar is a (re)emerging neglected tropical disease often associated with malnutrition. VL is a life-threatening infectious disease caused by protozoan of the Leishmania (Leishmania) donovani species complex, causing 59,000 deaths per year. Of the 500,000 annual cases, 90% occur in Bangladesh, Brazil, India, Nepal, Ethiopia and Sudan. VL provokes in the patients fever, severe cachexia, hepatosplenomegaly, pancytopenia and hypergammaglobulinaemia [10]. In most endemic areas children of young age are the most affected and immature immune system and malnutrition were documented as predisposing factors [11, 12]. A recent study confirms that percentages of VL patients with underweight ranges from 21.4% in Brazil to 92.6% in Sudan [13]. PEM was also shown to be a determinant factor for progression to clinical VL [14], and it is associated with more severe form of VL and a major risk for poor treatment outcomes [15].

The immunodeficiency caused by malnutrition is responsible for the high susceptibility to VL, but the specific mechanism underlying the interaction between VL and malnutrition are not totally understood. A protective immune response in human VL is associated with a predominant cell mediated immunity and a type 1 cytokine profile [16]. Different studies demonstrated a significant decrease of peripheral blood CD3+ cells, impairment in the development of effector cell responses and an alteration in the balance of type 1/type 2 cytokine expression in malnourished children as compared to the non-malnourished once [17, 18]. Moreover, a bias to T helper 2 (Th2) type responses in malnourished infected children with decreased IFN-γ and an increased IL-4 and IL-10 production was documented [19].

Studies of malnutrition in animal models have reported a decrease in the percentage of CD3+ T lymphocytes in the spleens of moderately and severely malnourished rats [20], and it has also been shown that the T cells ($CD4^+$ and $CD8^+$) from malnourished mice are quiescent [20, 21]. Furthermore, Anstead et al. [22] in experimental VL showed that early visceralization of *L. donovani* in malnourished BALB/c mice is due to failure of lymph node barrier function, and may be related to decreased levels of IL-10 and nitric oxide (NO) and excessive production of prostaglandin E2 (PGE2), an important negative regulator of host immunity.

Understanding the impact of the immunodeficiency caused by malnutrition in a population at risk for VL will help in the control effort; preventing the spread to new areas and/or mitigating the problem in affected communities. Libo Kemkem (Amhara, Ethiopia) is a high land district that converted to a low transmission area of VL after the outbreak detected in 2004/2005 [23, 24]. A study assessing the post outbreak situation indicated that children of 3 to 15 age group accounted for 30% of the cases. Moreover, 18% of the outbreak cases were malnourished [24]. In order to understand the effect of nutritional status on immunity we evaluate hematological parameters and levels of peripheral blood lymphocyte subsets, as well as the functional capability of these cells to produce cytokine, in malnourished and non-malnourished children of 4 to 15 years of age living in the new outbreak zone. In addition, we have measured serum levels of PGE2 in these children to assess the possible role of this factor in immunodeficiency and susceptibility to *Leishmania* infection.

Materials and methods

This study was done as part of an ongoing longitudinal study on childhood malnutrition and risk factors for asymptomatic VL, in the frame of the UBS-Optimus Foundation granted project "*Visceral leishmaniasis and malnutrition in Amhara State, Ethiopia".*

STUDY SUBJECTS. The study was conducted in children of age 4 to 15 years and was approved by the ethical review boards of Instituto de Salud Carlos III, Armauer Hansen Research Institute and the Ethiopian National Ethical Review Committee. Support letters were obtained from the Amhara State and the different districts' Health Bureau. Parents /guardians gave written informed consent prior to the enrolment of their children in the study, and for children above 11 years of age verbal assents were also obtained in addition to the consent of their parents/guardians.

The study was carried out in two phases. The first phase was done during May and July 2009, and included 456 children whose nutritional status, hematological parameters, and peripheral blood lymphoid subset were analyzed. The second phase was done during May and June 2010, enrolling 410 children, in whose nutritional, cytokine and serum PGE2 analyses were carried out. Anthropometric and clinical data were documented using structured questionnaires. Trained health professionals conducted the physical examination, anthropometric measurements (height, weight and age), clinical assessments and biological sample collection.

NUTRITIONAL STATUS. All children were measured and weighted according to standard WHO procedures (WHO Working Group, 1986). Malnutrition was defined according to the Body Mass Index (BMI) for Age in relation to the 2006 WHO Growth Standards for children ≤ 5 years and to the 2007 WHO Growth Reference for children older than 5 years respectively [25]. Global malnutrition was defined as BMI for Age Z score (BAZ) < -2 SD, moderate malnutrition as BAZ < -2 SD and BAZ > = -3 and severe malnutrition as BAZ < -3 SD.

HEMATOLOGICAL ANALYSIS. Blood samples from 394 children of the first phase were collected in 4 ml Na_2-EDTA tubes (SIGMA, UK) for hematological analysis. Analysis was done with hematology analyzer (Abbot CELL-DYN® 1800, USA) at the Amhara Health Bureau Regional Laboratory in Bahir-Dar.

CELL STAINING. Percentage of peripheral blood CD4+, CD8+ and CD19+ cells were determined by cell staining of whole blood samples and flow cytometry analysis. One hundred

μL. of blood was incubated with anti-human anti-CD4 FITC conjugated, anti-CD8 PE conjugated and anti-CD19 FITC-conjugated monoclonal antibodies (Immunotools – Germany), and red blood cells were lysed using lysis buffer (BD, USA). The stained cells were fixed with 2% formaldehyde and kept at 4^{o}C. Analysis with four colors FACSCalibur (BD, USA) was done within one week period of the cell staining date. Absolute number of cell subsets was determined taken into consideration the percentage of the different cell subsets after gating for lymphocytes at the FSC-SSC dot plot and the absolute number of peripheral lymphocytes obtained at the hematological analysis.

CELL PREPARATION AND *IN VITRO* STIMULATION ASSAY: Blood sample collected in 2 ml Na_2-EDTA tubes (SIGMA, UK) from 88 randomly selected children were used for *in vitro* stimulation assay with Phytohemaglutinin (PHA). One mL of whole blood was incubated for 15 minutes at 4 ^{o}C after gentle shaking with 1 mL ice cold ACK buffer to lyse red blood cells. After 5 minute centrifugation at 6000 rpm the supernatant was removed and the pellet washed in 2 mL PBS (7.2 pH). The washed pellet was diluted in 1 mL of complete medium (RPMI10 with 10% FCS complemented with antibiotics (100 IU/mL Penicillin and100 μg/mL Streptomycin) and subsequently 0.5 mL of the suspension of peripheral blood mononuclear cells (PBMC) were cultured in a flat bottom 24 well-plate with PHA (0.08 pg/μL final concentration). The culture supernatants were collected after 24 hours and kept frozen at -20 ºC until analysis.

DIRECT AGLUTINATION TEST. Two drops of blood were impregnated on Whatman 3MM filter paper (Whatman International Ltd., England), left to air dry and placed individually in sealed plastic bags. The dry blood was used to determine asymptomatic *Leishmania* infection and/or exposure to *Leishmania.* Direct agglutination test (DAT) with freeze dried antigen (ITMA-DAT/VL, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium) was used according to the manufacturer's protocol and titers ≥1:3200 were considered positive [26, 27].

CYTOKINE ANALYSIS. The level of cytokines in the serum and culture supernatant were measured by commercially available kit for flow cytometric detection (Diaclone Research, Besancon, France) as per the manufactures instruction.

PROSTAGLANDIN E2 ANALYSIS. Venous blood sample was collected in 5 mL plane tubes (SIGMA, UK) and serum was separated and stored at -20^{o}C. PGE2 assay was done for 94 randomly selected serum samples from DAT negative children. The ELISA assay was done using commercially available kit (Parameter™, R&D Systems, Inc, USA) according to the

manufacture's instruction. Cytokine and PGE2 concentrations (picogram per milliliter) were calculated using a standard curve developed with the standard provided with the kits.

STATISTICAL ANALYSIS. Comparison of mean values of cytokines, PGE2 and hematological parameters was performed using STATA version 11 (Stata Corp., College Station, TX, USA) and P values ≤ 0.05 were considered statistically significant.

RESULTS

Nutritional status

A total of 456 children were enrolled into the first study, of those 227 were boys and 229 were girls. In terms of nutritional status; 77.2% (352/456) were non malnourished and 22.8% (104/456) had acute malnutrition. Of the acutely malnourished children 5.0% (23/456) had the severe form and 17.8% (81/456) had the moderate form. Of the 410 (201 boys and 209 girls) children enrolled in the second phase; 77.1% (316/410) were non malnourished and 22.9% (94/410) had acute malnutrition. Of the acutely malnourished, 4.4% (18/410) had the severe form and 18.5% (76/410) had the moderate form.

Hematological analysis

We managed to get blood from 394 out of 456 children. Table 1 shows the red blood cell (RBC), Hemoglobin (HGB), hematocrit (HCT), and white blood cells (WBC), lymphocyte and subpopulations (CD4+, CD8+ and CD19+) of lymphocytes counts by nutritional status and sex. No difference was seen with sex and within the different nutritional status group of the girls in mean RBC, HGB and HCT. However, severely malnourished boys had significantly less RBC and HCT as compared to the normal and moderately malnourished ($P<0.05$) and their HCT was lower than the normal reference range. Moreover, their hemoglobin was less than the normal reference range but it was not significantly ($P=0.064$) lower as compared to the normal and moderately malnourished boys. Statistically significant difference was observed between the different nutritional status groups for total count of WBC, lymphocytes and sub populations of lymphocytes ($P <0.05$). There was no statistically significant ($P=0.161$) difference in CD4/CD8 ratio among the different nutritional status groups. The malnourished children had less CD19+ cells ($P=0.018$) as compared to the non-malnourished group (Table 1).

Cytokine production by PHA-stimulated PBMCs

Of the 88 samples used for PHA stimulation 57 were from non-malnourished, 21 from moderately and 10 from severely malnourished children. In the overnight PHA culture of PBMC, there was no detectable level of IL-4 and IL-12 in the severely malnourished children. Statistically significant level (P=0.002) of IL-10, TNF-α and IFN-γ were produced by the PBMC from the non-malnourished and moderately malnourished children as compared to the severely malnourished group. Higher level of TNF-α, IL-4, IL-10, and IFN-γ were detected in the supernatant of the PBMC culture from the non-malnourished children but the difference was not statistically significant (P>0.05) as compared to moderately malnourished children. There was no detectable level of IL-12 in the culture supernatant from the moderately malnourished children (Figure 1).

Serum levels of Prostaglandin E2 and Cytokines

From the total of 94 DAT negative serum samples assayed for PGE2; 61 were from non-malnourished, 6 were from severely malnourished and 27 were from moderately malnourished children. The mean PGE2 level was higher in the malnourished children as compared to non-malnourished. Significantly (P=0.007) high level was produced by the moderately malnourished children compared to the non-malnourished children (Figure 2).

Of the 43 DAT negative children; 29 were non-malnourished, 12 moderately malnourished and 2 severely malnourished while from the 36 DAT positive children; 18 were non-malnourished, 10 moderately malnourished and 8 severely malnourished. We observed difference in the cytokine production profile in the DAT positive and negative groups. In the DAT negative groups there was detectable level of all the assayed cytokines (IL-4, IL-10, IFN-γ and TNF-α) in non-malnourished children, but none in the severely malnourished children, and in the moderately malnourished children low level of IL-4 and IL-10 were detected. In the DAT positive groups there was significantly high (P=0.00) level of IFN-γ production in the non-malnourished when compared to malnourished children, higher level of TNF-α, IL-4 and IL-10 were produced by the non-malnourished children but was not significantly high compared to the moderately

malnourished children. Only IL-10 reached at a detectable level in the severely malnourished children (Fig 3).

DISCUSSION

The strong association between malnutrition and infections has been established through epidemiologic studies conducted in several different countries. The severity of malnutrition determines the risk of death and/or severity of infections [2, 4, 6, 14]. In our study area, 18% of the visceral leishmaniasis cases were reported to be malnourished and malnutrition was one of the implicated risk factors for the outbreak [24]. In line with this we assessed the effect of malnutrition on hematological parameters and cytokine production.

Our data showed significantly lower total count of white blood cells, lymphocytes and sub populations of lymphocytes (CD4+ and CD8+ cells) in severely malnourished children ($P < 0.05$). Hematopoietic tissue requires a high nutrient supply, and in consequence bone marrow function may be altered by nutritional deficiencies that can be reflected in a change of the hematological parameters (RBC, WBC, etc…). A similar observation was reported in animal model study; malnourished mice presented anemia with reduced hemoglobin concentration, and total number of erythrocytes, leucopenia with depletion of polymorphonuclear granulocytes, lymphocytes and monocytes [29]. Change in the hematopoietic environment (high fibronectin and laminin) and biochemical evidence of alterations in extracellular matrix proteins in the bone marrow was observed in malnourished mice [30, 31]. And severe malnutrition during childhood was shown to affect thymic development, which compromises immunity by a long-term reduction of peripheral lymphocyte counts [15].

The absence of significant difference in the CD4+/CD8+ ratio in our data indifferent to the report by Nassar et al 2007 [32], supports the idea that the immunodeficiency associated with the severe form of malnutrition is not due to increased helper-suppressor T-cells ratio[33].

Our stimulation data showed that the PBMC from the severely malnourished children produced significantly low or no cytokine response to PHA. This probably means that the cells from the severely malnourished children are non-responsive (quiescent). With intracellular staining and flow cytometric analysis Rodriguez et al 2005 [34] reported significantly lower CD4+ IL-2-positive cells in malnourished children compared to those in well nourished children. In addition, they showed that cells from malnourished children had impaired activation capability

as compared to the well-nourished, infected children. A reduction in the number of lymphocyte subpopulation producing a specific cytokine or the dormancy induced by malnutrition could alter the capacity of cells to produce specific cytokines in response to a stimulus.

The high level of PGE2 in the serum of the DAT negative moderately malnourished children observed in our data is in accordance with previous observation. PGE2 production is enhanced in malnutrition [22, 35]. Also, Anstead *et al*. [22] demonstrated that mice fed with a diet deficient *in* protein, iron and zinc have an altered innate immune response with lymph node cells from malnourished infected mice producing increased levels of PGE2, decreased levels of IL-10 and had a lower inducible nitric oxide synthase (iNOS) activity in the spleen and liver.

The serum from both the DAT positive and the DAT negative non-malnourished children produced a higher level of cytokines than the malnourished. In the DAT negative group, the severely malnourished children produced no detectable level of any of the cytokines assayed; the moderately malnourished children produced IL-4 and IL-10, while the non-malnourished groups produced all the assayed cytokines (Fig2). The bias to the Th1 type in the non-malnourished DAT negative children and Th2 type in the moderately malnourished group is in agreement with our PGE2 data explained above (Fig 1). Previous *in vivo* studies have also demonstrated that PGE2, can affect cytokine production directly during an inflammatory response; the production of Th1cytokines (IFN-γ and TNF-α) are down regulated [36].

The DAT positive non-malnourished children produced significantly high level of IFN-γ and IL-10 as compared to the moderately and severely malnourished children (Fig 3). Our data is in agreement with behavior of immune response in *L. donovani* infection; IFN-γ is secreted at the very initial stages of the exposure to the parasites as observed in the seroconverted or subclinically infected individuals in the endemic area [37]. Carvalho et al. [38] observed high levels of IFN-γ in oligosymptomatic individuals who evolved to spontaneous cure, supporting the fact that resistance is related to an efficient cellular immune response. Also Gama et al [39] described the detection of IL-12 in 85.2%, IFN-γ in 48.1%, IL-10 in 88.9%, and TNF- α in 100.0% subclinically infected children. However the detection of low level of IL-4 in our non-malnourished DAT positive children might indicate that some children might shift the Th2 response and progress to the clinical from of VL. While the detection of the low IFN-γ and relatively high IL-4 and IL-10 in the moderately malnourished children indicates a Th2 bias in this group that might be related to the high level of PGE2 production.

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Table 1: Comparison of the mean hematological parameters in children of different nutritional status groups, Libo Kemkem, Ethiopia.

Parameters Mean ±SE	Nutritional Status (N=number per group)			Reference range*[1,2]
	Normal	Moderate	Severe	
RBC (10^6 cells/µL)				
Boys	4.55±0.42 (134)	4.58±0.08 (42)	4.09±0.21 (12)	3.80-6.20
Girls	4.50±0.04 (170)	4.55±0.08 (27)	4.34±0.15 (9)	3.80-5.60
HGB (g/dL)				
Boys	12.74 ± 0.14 (134)	12.80±0.21 (42)	11.63±0.61 (12)	12.20-17.70
Girls	12.78±0.15 (170)	12.75±0.32 (27)	12.41±0.42 (9)	9.50-15.80
HCT (%)				
Boys	37.43±0.34 (134)	37.70±0.72 (42)	34.20±1.83 (12)	35.00-50.80
Girls	37.04±0.41 (170)	37.17±0.90 (27)	36.07±1.19 (9)	29.40-45.40
WBC (10^3/µL	8.03 (304)	7.01 (69)	5.67 (21)	3.10-9.10
LYMP (10^3/µL)	3.63 (304)	2.97 (69)	2.39 (21)	1.20-3.70
CD4 (10^3/µL)	1.09 (124)	0.99 (23)	0.64 (10)	0.457-1.628
CD8 (10^3/µL)	0.91 (124)	1.05 (23)	0.53 (10)	0.23-1.178
CD4/CD8 (ratio)	1.38 (124)	1.42 (23)	1.30 (10)	
CD19 (10^3/µL)	0.34 (124)	0.26 (23)	0.17 (10)	

*1=Establishing Clinical laboratory Reference Intervals in Africa; IAVI (International AIDS Vaccine Initiative) and *2= Immunohematological Reference Ranges for Adult Ethiopians [28].

FIGURE LEGENDS

Figure 1: Cytokine Concentration (pg/ml) in a supernatant from a 24 hour culture of peripheral blood mononuclear cells with PHA, in children from Libo Kemkem, south Gondar, Ethiopia. Cytokine measurement was performed for 57 non-malnourished, 21 moderately malnourished and 10 severely malnourished children.

Figure 2. Serum concentration of PGE2 by nutritional status in children from Libo Kemkem, South Gondar, Ethiopia: Serum samples from 94 DAT negative children; 61 from non-malnourished, 6 from severely malnourished and 27 from moderately were analyzed.

Figure 3. Serum concentration of cytokines (TNF- α, IL-10, IL-4, and IFN- γ) by nutritional status and asymptomatic infection as detected by DAT positivity in children from Libo Kemkem, South Gondar, Ethiopia; 43 DAT positive (29 non malnourished, 12 moderately malnourished and 2 severely malnourished) and 36 DAT positive (18 non malnourished, 10 moderately malnourished and 8 severely malnourished) serum samples were analyzed.

FIGURE 1

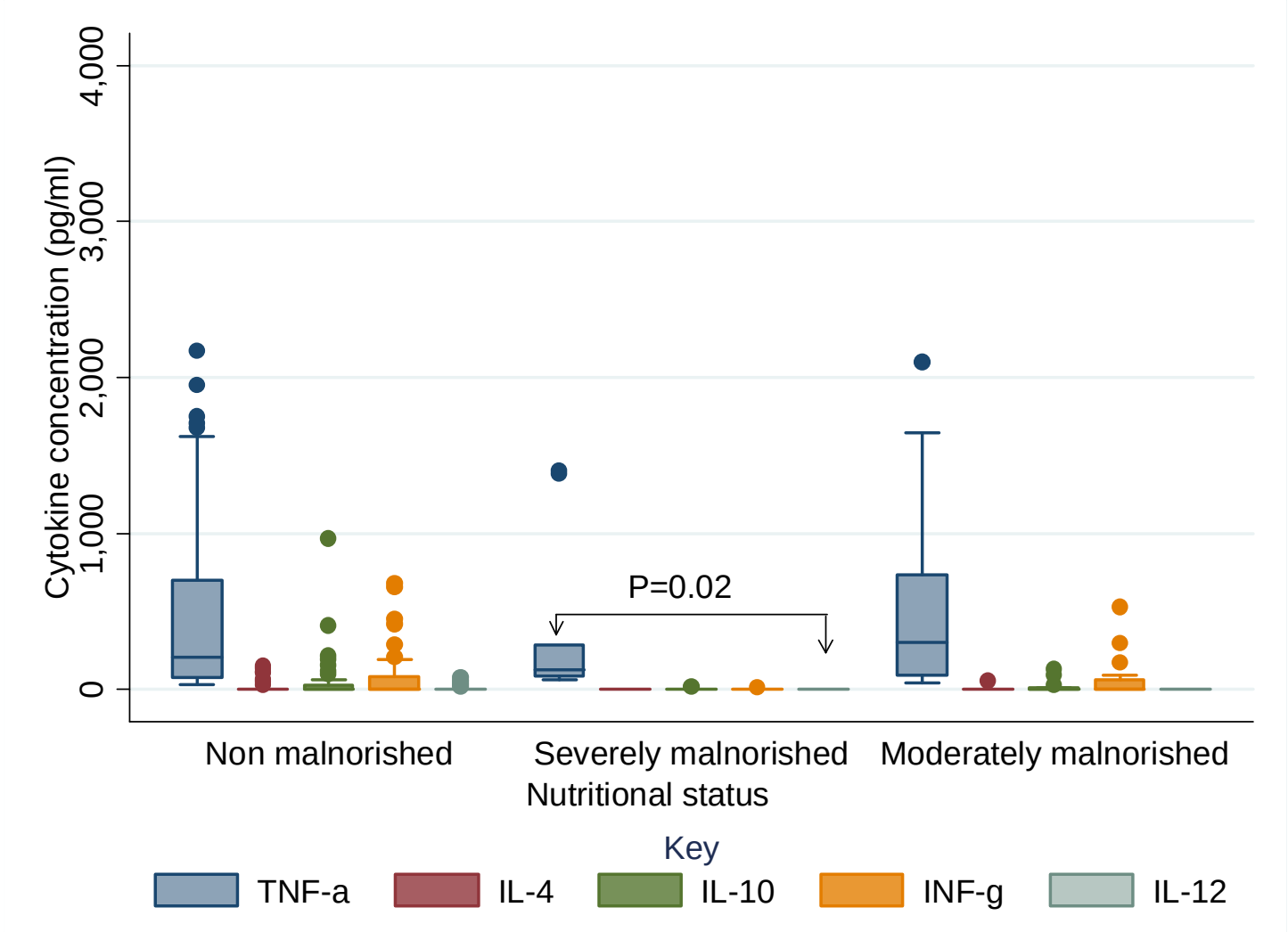

Cytokine concentration (pg/ml)
0
1,000
2,000
3,000
4,000
P=0.02
Non malnorished
Severely malnorished
Moderately malnorished
Nutritional status
Key
TNF-a
IL-4
IL-10
INF-g
IL-12

FIGURE 2

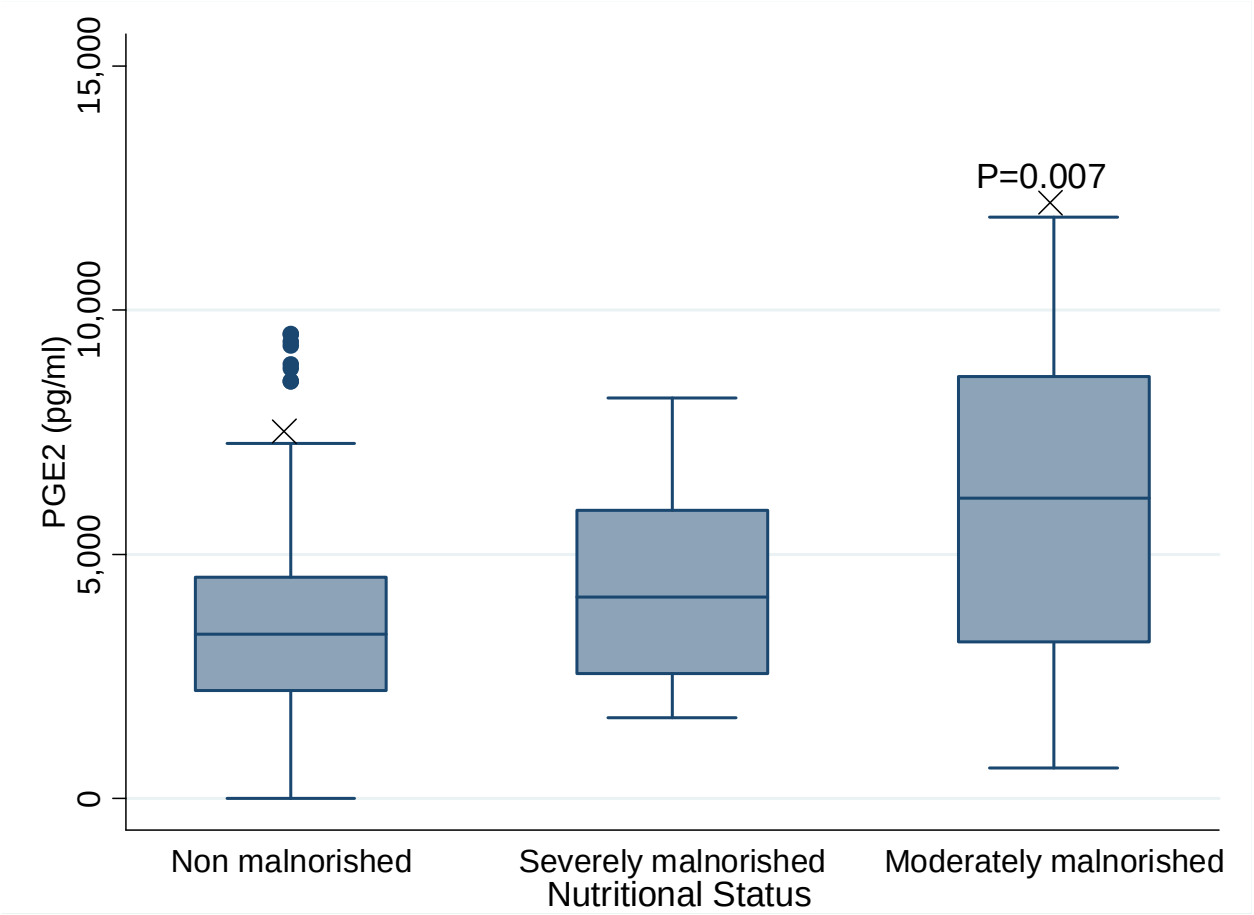
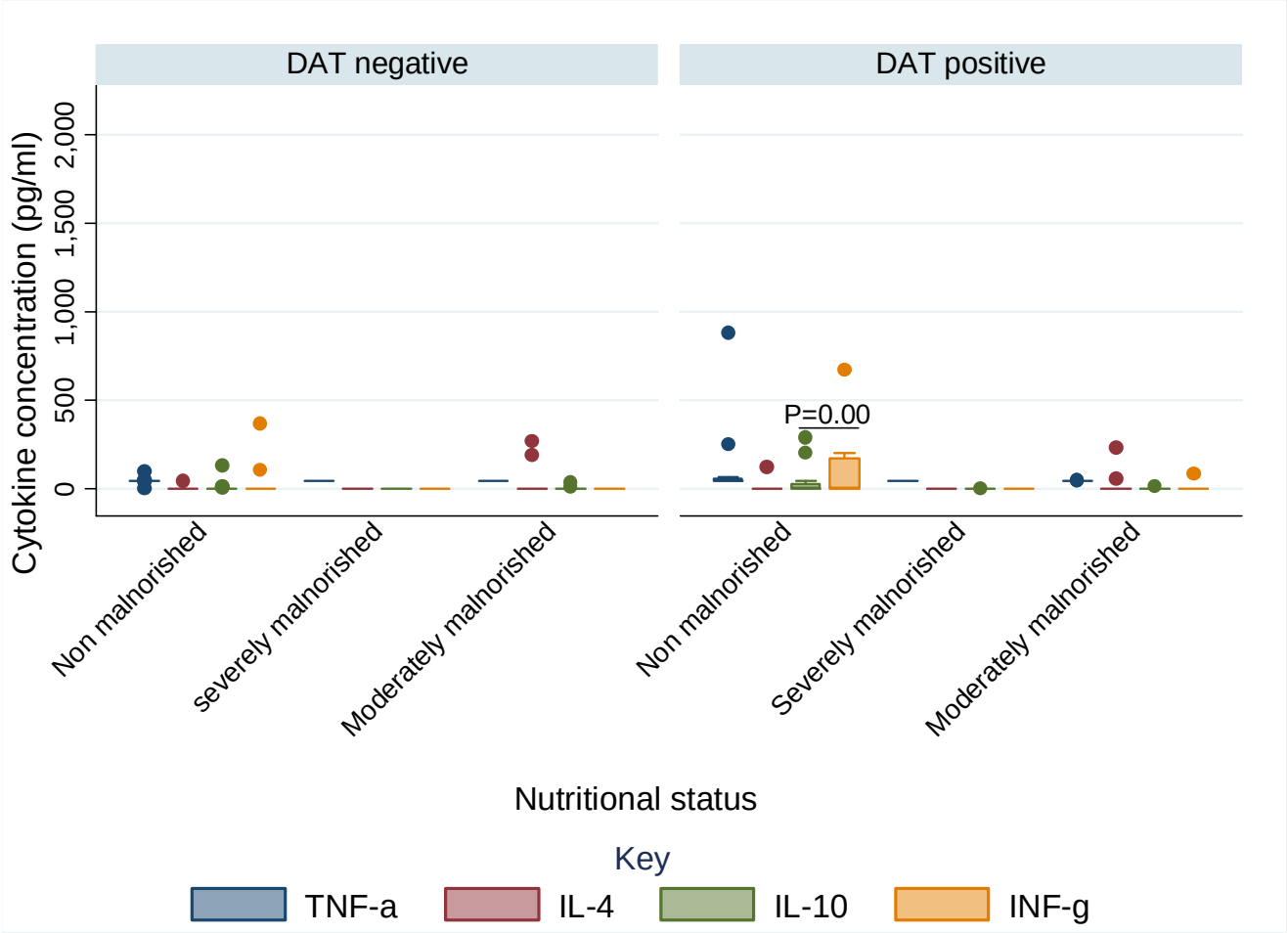

PGE2 (pg/ml)
0
5,000
10,000
15,000
P=0.007
Non malnorished
Severely malnorished
Moderately malnorished
Nutritional Status

FIGURE 3


DAT negative
DAT positive
Cytokine concentration (pg/ml)
0
500
1,000
1,500
2,000
P=0.00
Non malnorished
severely malnorished
Moderately malnorished
Non malnorished
Severely malnorished
Moderately malnorished
Nutritional status
Key
TNF-a
IL-4
IL-10
INF-g

VIII- DISCUSIÓN

En Etiopía, la LV está distribuida en todas las tierras bajas con diferentes grados de endemicidad. Se estima una incidencia de 4.500 a 5.000 casos nuevos por año y la tasa de coinfección *Leishmania*/VIH está entre el 15% y el 30% de todos casos de LV (Alvar *et al*., 2006). En los últimos años la LV se ha extendido en Etiopía, se han documentado nuevos brotes en el norte y sur del país: Libo Kemkem y Belesa (región de Amhara), Shiraro (región de Tigray) e Imey (región de Somalí) (Informe de Ministerio de Sanidad de Etiopía). Se piensa que los movimientos de trabajadores temporales a las zonas endémicas, la malnutrición asociada a la pobreza y la coinfección *Leishmania*/VIH han contribuido a la expansión de la LV a zonas previamente no endémicas (Herrero *et al*., 2009). Como consecuencia de este incremento la LV se ha convertido en una preocupación para la salud pública del país y su control se ha convertido en una prioridad. Para plantear un programa de control frente a la LV adaptada a las situaciones locales, hay que disponer de datos socio-epidemiológicos exhaustivos de buena calidad que permitan monitorizar el progreso del programa y la evaluación de su impacto. El entendimiento de la influencia de factores de riesgo como la malnutrición, y la disponibilidad de herramientas valoradas localmente para establecer las situaciones epidemiológicas tendrán un impacto positivo en los esfuerzos realizados para el control de la LV.

El presente estudio mostró la presencia de infección asintomática por *Leishmania* en los niños de entre 4 y 15 años de edad en los distritos de Libo Kemkem y Fogera, en el nuevo foco del región de Amara, noroeste de Etiopía. La tasa global de la infección asintomática (positiva por LST y/o serología) en

los comunidades en que se han documentado mayor número de LV después del brote (2008) fue del 10,1% (61/605), el 6,3% fueron individuos seropositivos y el 5,6% fueron positivos para LST. La discordancia observada entre LST y serología son probablemente debido a la diferencia del tipo de respuesta inmune detectada por cada prueba. LST mide la reacción de hipersensibilidad retardada frente *Leishmania* mientras que la seropositividad resulta de la respuesta de anticuerpos específicos a *Leishmania*. La positividad de LST aparece más tarde después de la infección, mientras que la seropositividad es considerada como un marcador de una infección más reciente. Por la misma razón, el LST positivo se produce como resultado de la exposición repetida a la infección natural, lo que también justifica porqué el número de individuos positivos para LST es mayor en los grupos de mayor edad. La mayor tasa de infección detectada por serología (con DAT y rK39-ICT) al comparar con las obtenidas con LST puede estar asociado a la necesidad de periodos de tiempo más largos para el desarrollo de respuesta positiva en el LST con respecto a la seropositividad y a que la LV ha aparecido recientemente en nuestro área de estudio. La diferente tasa de infección observada entre las pruebas serológicas puede depender de la etapa de la enfermedad/infección, la baja tasa detectada por la prueba rK39-ICT puede ser explicada por su capacidad para detectar solo los anticuerpos específicos frente al antígeno rK39, mientras que el DAT detecta una respuesta de anticuerpos frente a una amplia gama de antígenos de *Leishmania* (el promastigote completo liofilizado) (Boelaert *et al*., 2004). Otra posible explicación se basa en la naturaleza de las pruebas; tal y como propusieron ter Horst *et al*., (2009) los anticuerpos detectados por rK39-ICT podrían tener una menor capacidad de reacción en el formato rápido de tira

inmunocromatográfica que en las incubaciones durante toda la noche usadas en el DAT. La observación de que la tasa de infección asintomática aumenta con la edad es consistente con un foco endémico de LV, de hecho, el incremento de edad es un factor del riesgo para la infección asintomática. La presencia de infección asintomática en individuos menores de 5 años de edad es consistente con la presencia de una transmisión activa. El mayor número de infección asintomática detectado por el uso combinado de las pruebas serológicas (particularmente DAT) y LST, indica que este método combinado es el procedimiento más apropiado para determinar la tasa de infección asintomática en un área endémica de LV. En efecto, la prevalencia de LV asintomática detectada varía según las técnicas y el área endémica por lo que se aconseja la combinación de técnicas para determinar la prevalencia más cercana a la realidad en ausencia de un método *Gold Standard* para la detección de infección asintomática (de Gouvea Viana *et al*., 2008).

La prevalencia global detectada en las áreas donde hubo al menos un caso clínico de LV durante el brote de 2004/2005 fue del 1,02%. Los niños mayores de 12 años presentaban la mayor prevalencia (2,56%), seguidos por el grupo de niños entre 8 y 11 años de edad (0,82%), y la menor prevalencia fue detectada en los menores de 8 años (0,49%). La baja prevalencia detectada en nuestro estudio indica que las condiciones que provocaron la epidemia de la LV en la región de estudio ya no persisten. Una hipótesis propuesta durante la epidemia de LV mantenía que el parásito había sido introducido en la región por trabajadores agrícolas, quienes habían regresado infectados a sus pueblos después de completar su trabajo estacional en la frontera de Sudán y habían actuado como reservorio del parásito (Herrero *et al*., 2009). Sin embargo, de

acuerdo con la información disponible, los afectados no fueron sólo estos trabajadores agrícolas desplazados, y no hay ninguna evidencia de que tal migración haya cesado. Esto nos hace pensar que algún cambio en la población del vector debió de provocar el brote, se ha sugerido que cambios en la temperatura y humedad relativa pueden aumentar la abundancia del vector (Gálvez *et al*., 2010).

Por otro lado, también es posible que la respuesta al brote de LV y el tratamiento de los enfermos pudiera haber llevado a la reducción de la tasa de seroprevalencia. Antes del año 2004 no se había reportado ningún caso de LV en Libo Kemkem, y probablemente se está volviendo a la situación pre-epidémica. No obstante, las dudas sobre las causas del brote y la caída de la prevalencia post brote que hemos observado en este estudio resaltan la necesidad de vigilar los cambios climáticos para evitar la reemergencia de la LV en esta zona.

Durante el brote de LV de 2004/2005, la prevalencia determinada por LST fue considerablemente mayor que la observada en nuestro estudio, 34% en hombres y 26% en mujeres (Alvar *et al*., 2007). No obstante, este estudio fue hecho para valorar el brote en la población de 0,7 a 60 años de edad, y se hizo mediante un muestreo por conveniencia.

La fuerte variación en la infección asintomática observada entre zonas altamente endémicas de LV está de acuerdo con la agrupación especial observada en otros lugares en estudios de infección asintomática (Evans *et al*., 1992; Singh *et al*., 2010) y de casos clínicos (Bern *et al*., 2005; Ryan *et al*., 2006).

El aumento de la edad constituye un factor de riesgo para la infección por *Leishmania* y se asocia con las actividades específicas de los niños mayores o adolescentes que implican un potencial aumento de la exposición a la picadura del flebótomo (Singh *et al*., 2010). Se piensa que esta situación es también responsable de la mayor frecuencia de infección entre el sexo masculino (Ali y Ashford, 1993). Nuestros resultados respaldan esta hipótesis, actividades tales como el hábito de dormir fuera de la casa y el cuidado del ganado se han identificado como factores de riesgo para la infección asintomática. La mayor exposición a los flebótomos durante el cuidado del ganado se puede asociar con estar en el campo al anochecer y amanecer, momentos del día en los que los flebótomos son más activos (Wijers, 1963) y también con una mayor proximidad a las acacias rojas (pudimos comprobar que el 82% de los niños que van a cuidar ganados descansaban a la sombra de las acacias rojas), que se consideran como sitios de descanso diurno para *Phlebotomus orientalis*, el potencial vector de *Leishmania* en el área de estudio (Elnaiem *et al*., 1999).

En nuestro análisis de factores de riesgo, la malnutrición aguda se asocia positivamente a la infección asintomática, pero solo en el análisis univariante. Cuando el sexo y la edad fueron introducidos en el modelo la malnutrición aguda perdió su significancia, probablemente reflejando la interacción entre las tres variables, ya que factores como el sexo masculino y el aumento de la edad mostraron una asociación directa y significativa con la malnutrición aguda. El riesgo de infección asintomática asociado con un mayor número de miembros en la familia podría ser debido a la atracción de los flebótomos por grandes biomasas, que se ha descrito como un riesgo de LV en

el mismo área (Bashaye *et al*., 2009). También había mayor probabilidad de infección asintomática entre los niños de una familia con historia previa de casos de LV en la familia, aunque esta asociación sólo fue significativa con los casos seropositivos y no con los positivos por LST, lo que está de acuerdo con las conclusiones de Bern *et al*. (2007) en Bangladesh. En relación con la seropositividad, otros estudios ya han mostrado la asociación entre este factor y el contacto previo con enfermos de LV (Caldas *et al*., 2002; Evans *et al.,* 1992; Schaefer *et al*., 1995), apoyando la hipótesis de transmisión dentro de la familia.

El incremento del riesgo de infección asintomática en familias que viven en una casa de techo de paja y con grietas en las paredes frente a las que viven en casas con chapa ondulada puede estar relacionado con el poder económico de la familia y refleja su pobreza/riqueza, además el techo de paja y las grietas en los paredes sirven como sitio potencial de reproducción y de descanso diurno por los flebótomos, incrementado su supervivencia y abundancia. No obstante, con respecto a *P. orientalis, se* necesitan más estudios para confirmar este último punto, ya que los pocos estudios existentes en la literatura señalan un comportamiento exofágico del vector, incompatible con este hipótesis (Gebre-Michael *et al*., 2010).

Por otro lado, la posesión de un mayor número de cabezas de ganado por familia mostró un efecto protector frente a la infección asintomática y seropositividad, pero no a la positividad por LST. El efecto protector del ganado y gallinas frente a la LV puede explicarse por su papel como indicadores de riqueza o también por el efecto zooprofiláctico del ganado (Caldas *et al*., 2002; Schenkel *et al*., 2006). La preferencia *P. orientalis* por la sangre del ganado

frente a la del hombre ya se ha demostrado en otra parte de Etiopía, contribuyendo a esta última teoría (Gebre-Michael *et al*., 2010), pero el efecto zooprofilático de las gallinas con respecto a *P. orientalis* necesita ser resuelta.

Con respecto a los efectos de la malnutrición sobre el status inmunológico de los niños, nuestro estudio mostró un número de leucocitos, linfocitos y células T CD4+ y CD8+ significativamente menor en los niños con malnutrición severa ($p < 0,05$). La malnutrición severa durante la infancia afecta el desarrollo del timo, lo que compromete la inmunidad a largo plazo y produce una reducción del número de linfocitos en sangre periférica. La baja o nula expresión de citoquinas observada en las células de los niños con malnutrición severa tras la estimulación con PHA está probablemente asociada con la menor actividad de estas células. Rodríguez *et al*. (2005) reportaron niveles significativamente menores de células T CD4+ e IL-2+ en niños malnutridos en comparación con los niños bien nutridos. Además, se demostró que las células procedentes de los niños malnutridos tienen deficiencia en el poder de activación en comparación con las células procedentes de niños bien nutridos.

Los mayores niveles de PGE2 observados en el suero de los niños DAT negativos y con malnutrición moderada está de acuerdo con observaciones previas en las que la producción de PGE2 aumenta con la malnutrición (Anstead *et al*., 2001; Dooper *et al*., 2002). Además, la predisposición a una respuesta de citoquinas tipo Th1 en los niños no malnutridos y DAT negativos, y de tipo Th2 en los moderadamente malnutridos está también de acuerdo con el dato de PGE2 explicado anteriormente.

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IX- CONCLUSIONES

Se han evaluado a nivel local herramientas diagnósticas para la determinación de infección asintomática sobre el terreno, aspecto clave a la hora de implementar un programa de control en áreas donde la LV es antroponótica. Se ha determinado la utilidad de combinar DAT y LST para obtener la mejor medida de la tasa de infección asintomática en la comunidad. Esta metodología puede apliacrse igualmente en otras áreas endémicas de Etiopía.

La prevalencia de LV activa y de infección asintomática en las poblaciones estudiadas fue baja, indicando la necesidad de identificar áreas con mayor prevalencia y enfocar el esfuerzo de control en ellas. Podemos considerar que la baja prevalencia post-brote puede deberse a la rápida instauración de un centro de tratamiento de LV, que contribuye a disminuir el número de individuos infectados (reservorios) en áreas de transmisión antroponótica. Se debe también considerar una posible disminución de la población de vectores a niveles similares a los de antes del brote. Una hipótesis de origen del brote es el aumento de la población de vectores en el área debido a cambios ambientales. No obstante, esto último no ha podidi probarse al no existir un sistema de vigilancia vectorial.

Los factores personales (comportamiento), medioambientales y socioeconómicos que hemos identificado como asociados a un aumento del riesgo de infección asintomática son una aportación indispensable a la hora de plantear un programa de control de LV.

A través del estudio de factores de riesgo y del estudio de los aspectos inmunológicos, hemos comprobado que la malnutrición influye en la infección por *Leishmania*. Por tanto consideramos que es necesario considerar el aspecto nutricional, particularmente en los grupos más vulnerables, a la hora de plantear un programa de control y prevención de la LV.

X - ANEXO I

Galeria de fotos

ÁREA DE ESTUDIO

El área de estudio es una meseta situada de 1800 a 2000 metros sobre el nivel del mar. Durante la estación de lluvias, (junio-septiembre) la mayor parte del área está inundada (1). A partir de octubre se empieza a secar y entre diciembre y febrero, la estación seca (2), se forman grietas profundas en el suelo que constituyen lugares ideales para el refugio, durante el día, de los flebótomos (3)

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Debido a las actividades agrícolas la cobertura vegetal del área se ha visto reducida y por ello la principal fuente de energía para cocinar son los excrementos secos de vaca. Alrededor de numerosas viviendas, ya sea de tejado de paja o de chapa ondulada, pueden encontrarse apiladas las heces secas de vacas (4 y 5). Además, los animales se mantienen cerca (6) o incluso dentro de las viviendas (7).

POBLACIÓN DE ESTUDIOS

En este estudio se incluyeron niños y niñas de 4 a 15 años de edad residentes en diferentes *kebeles* (sub-distritos) de Fogera y Libo Kemkem (8, 9 y 10)

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COMIDA

La *injera* es una torta fina hecha de harina de *teff* fermentada (11), o de una mezcla de harina de teff y de otros cereales. Se le suele añadir diferentes tipos de puré de legumbres (*shiro*), y verduras u hortalizas como tomate, patata, zanahoria o pimientos (12). En el *shiro* se añade aceite o a veces manteca, muy ocasionalmente se añade carne. El *teff* es el principal cultivo de la zona (13).

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ENTRENAMIENTO

Antes de realizar el trabajo de campo se organizó un entrenamiento de los enfermeros encargados de realizar las encuestas, las medidas antropométricas, la toma de muestras biológicas y los diferentes análisis que se realizaron *in situ*. Entrenamiento en la prueba serológica DAT (14) y en la toma de medidas antropométricas en el aula del hospital de Bahir Dar (15). Entrenamiento en la prueba serológica rK39-ICT (16) y en la intradermorreación de la leishmanina (17).

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ESTUDIO PILOTO

Se realizó un estudio piloto para optimizar los métodos de colección de los datos y confirmar las habilidades prácticas de los enfermeros. Personal experto acompañó al grupo de enfermeros al campo para asesorar y evaluar su trabajo práctico a la hora de hacer las medidas antropométricas (18) y las pruebas diagnósticas (19). Los problemas y resultados de estudio piloto se pusieron en común en una reunión final (20).

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TRABAJO DE CAMPO

En el momento de realizar el trabajo de campo, el equipo de encuestadores se instalaba en una zona de cada uno de los pueblos seleccionados (*gotts*) a la que acudían los niños para su examen (21). El examen físico incluía medida de temperatura (22), peso (23), talla (24) y la toma de sangre (25). A la vez se realizaba una encuesta al adulto responsable de los niños (26)

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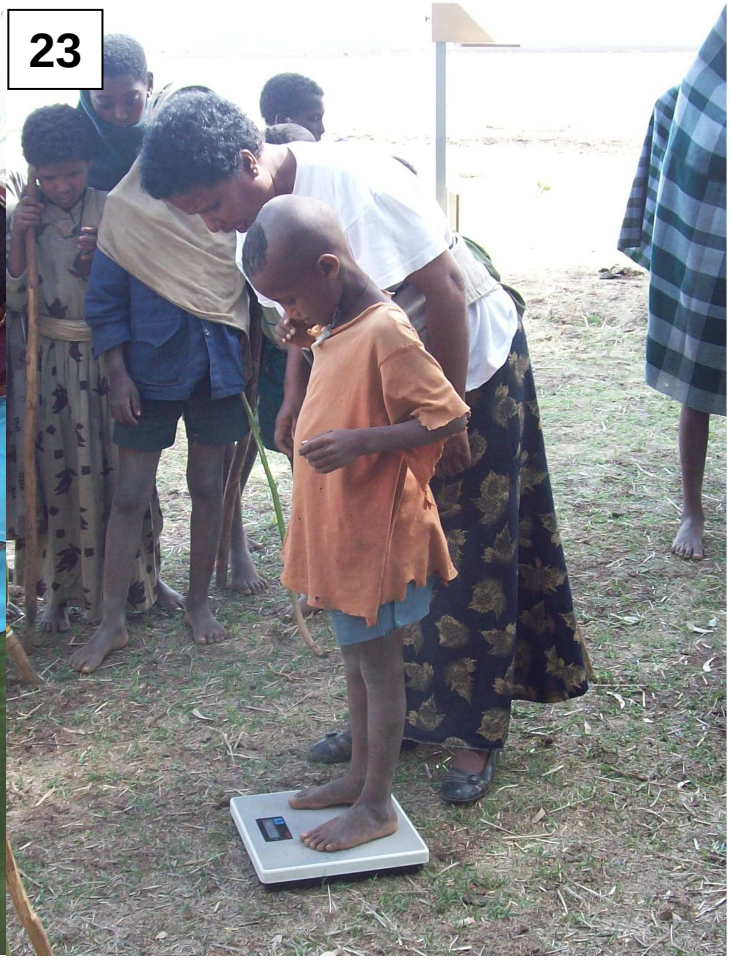

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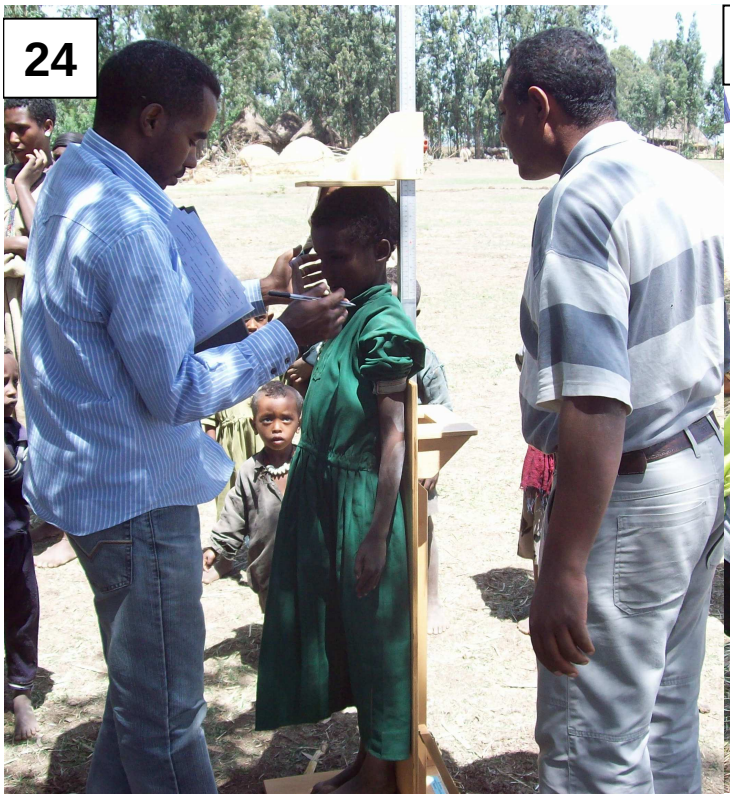

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Durante el estudio transversal se realizó a los niños la prueba de la leishmanina. Para ello se inoculaba por vía intradérmica, 100 µL de la solución de leishmanina (27), y a las 42 – 72 horas se midió la presencia de induración mediante el método del bolígrafo (28). Esta prueba es positiva si el diámetro de la induración es mayor de 5 mm (29), en caso contrario se considera negativa (28) .

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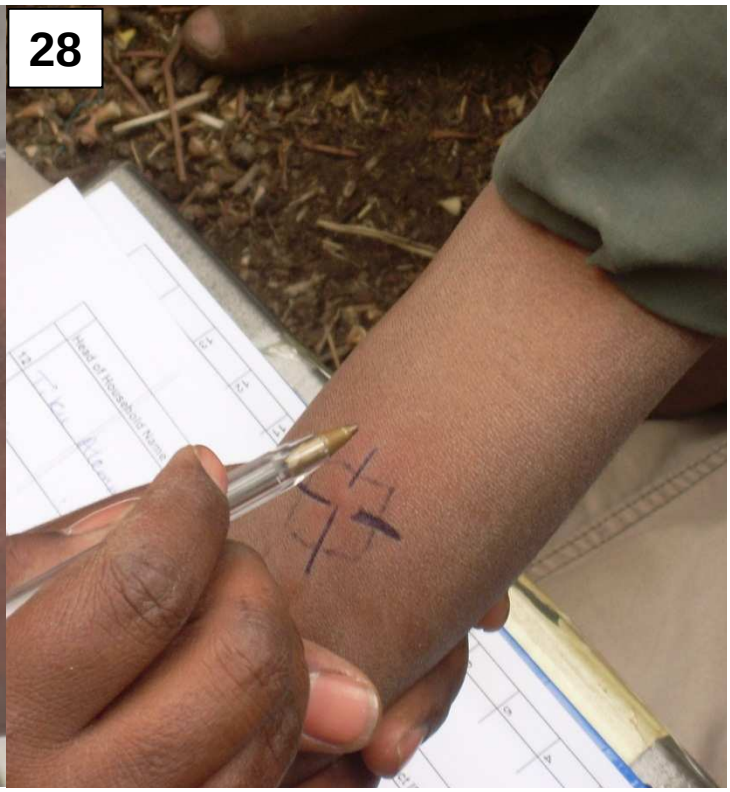

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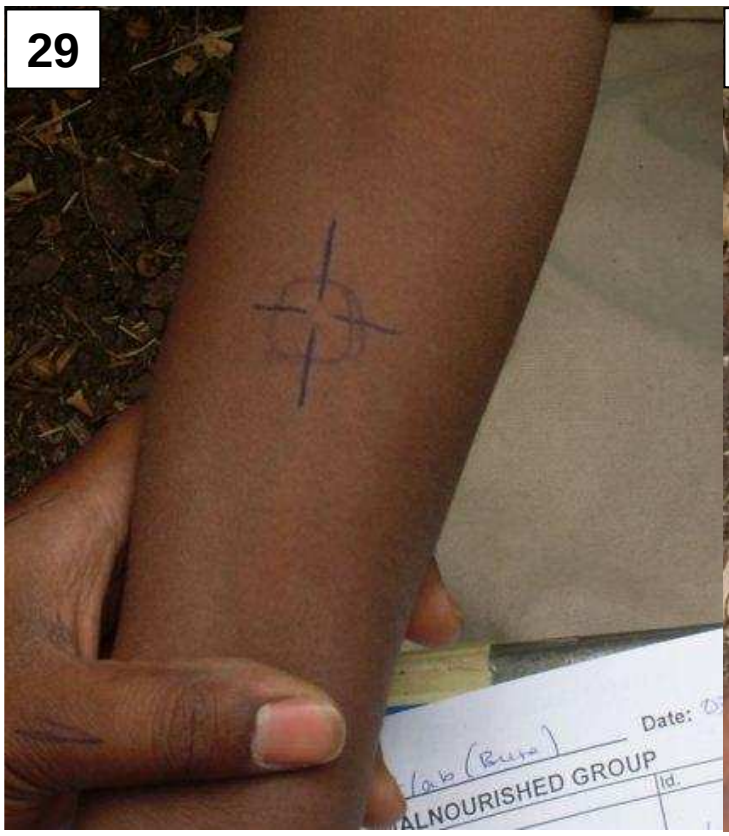

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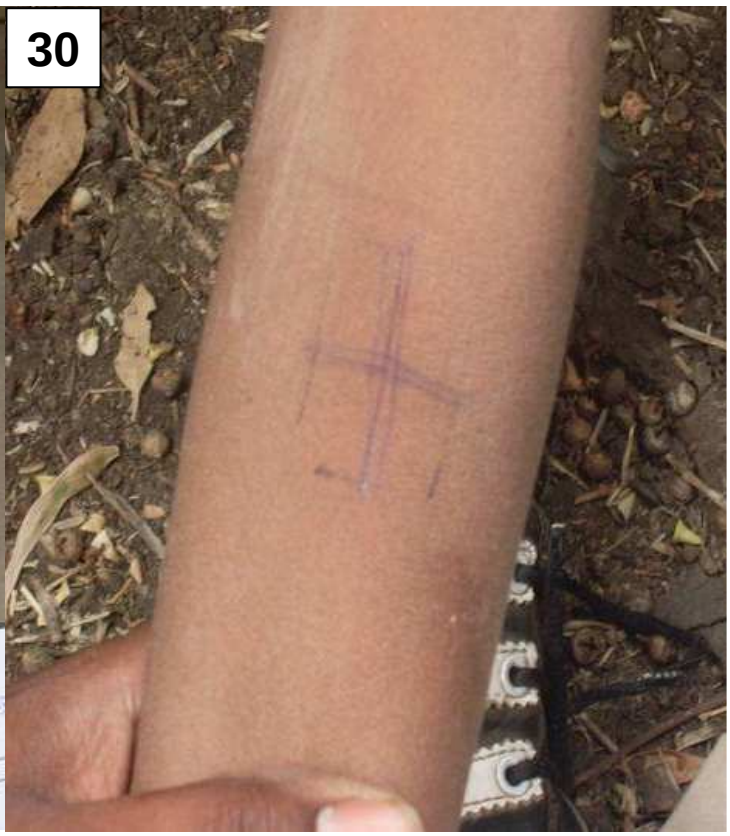

TRABAJO DE LABORATORIO.

Las muestras obtenidas durante el trabajo de campo se trasladaban el mismo día al Laboratorio Regional de Amhara, en Bahir Dar, para su procesamiento (31). En este laboratorio se realizaba el hemograma (32), separación, cultivo y tinción de linfocitos (33 y 34), la separación del suero y el test DAT.

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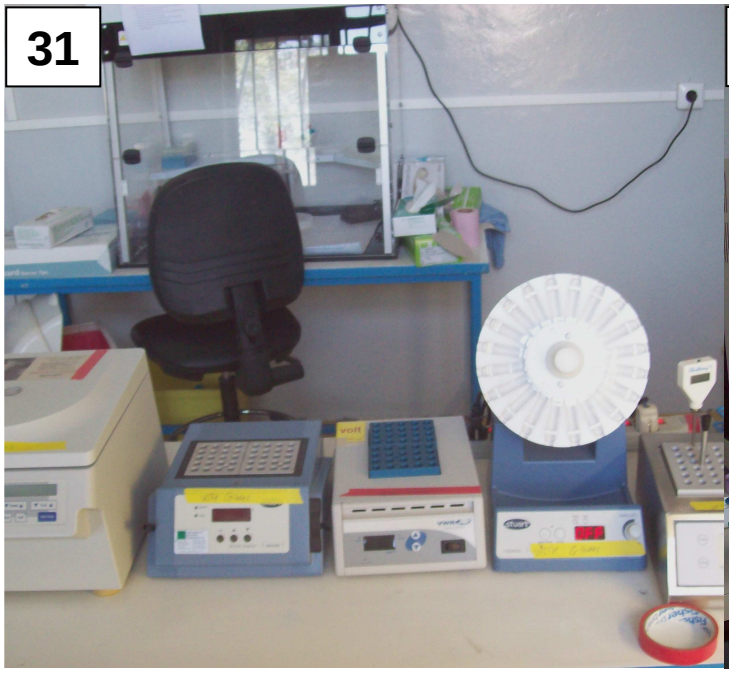

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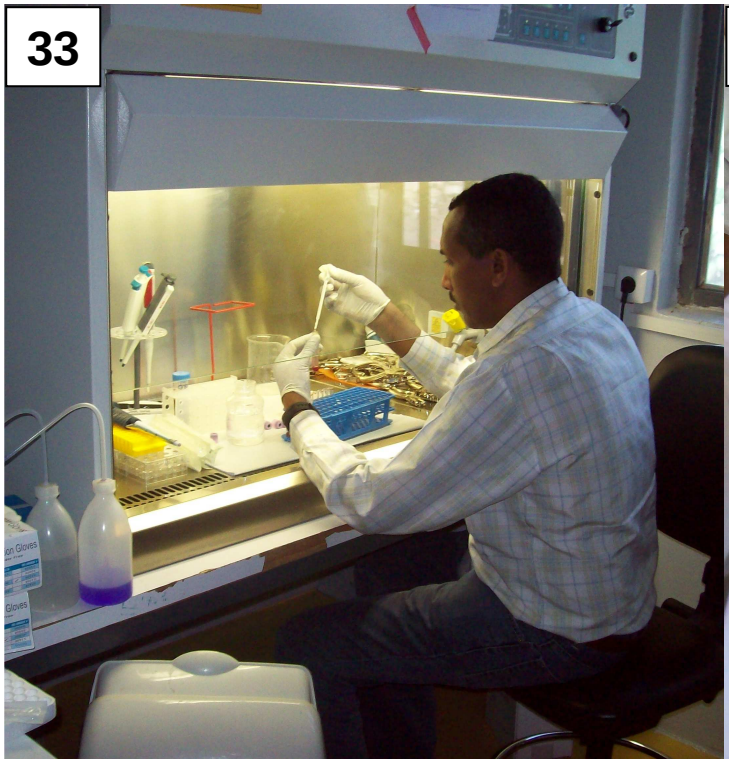

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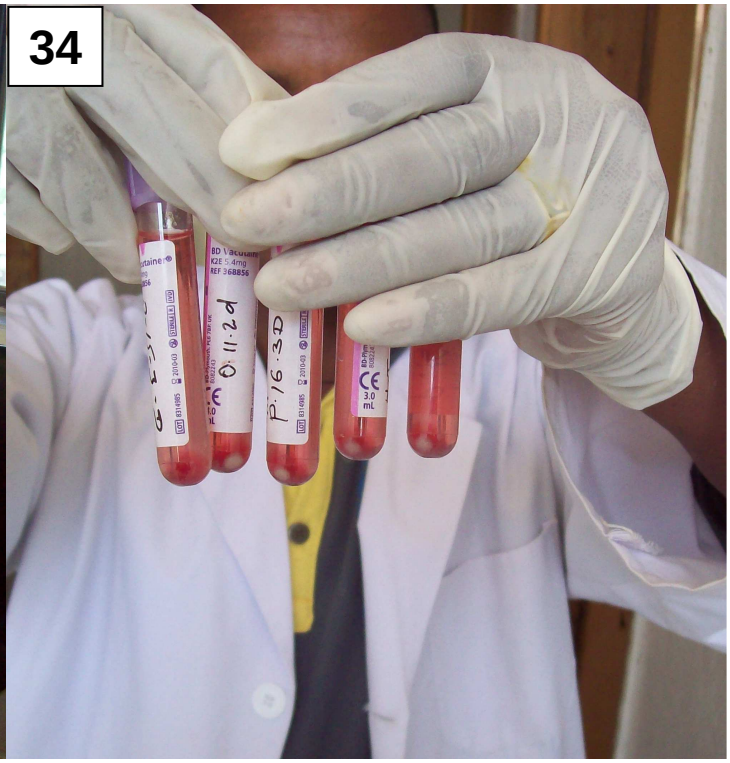

EVALUACIÓN EXTERNA DEL PROYECTO

En noviembre de 2009 se llevó a cabo una evaluación del proyecto por parte de un experto externo, el Dr. Philippe Dexjeux , Institute for One World Health), quien visitó los diferentes centros implicados en el proyecto y el área de estudio (35, 36 y 37).

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